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## Abelson Kinase Based Regulation of Tumor Cell Invasion in HNSCC

Karen E. Hayes  
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# **Abelson Kinase Based Regulation of Tumor Cell Invasion in HNSCC**

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Dissertation Submitted to the School of Medicine at West Virginia University  
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Cancer Cell Biology

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## **Abstract**

### Abelson Kinase Based Regulation of HNSCC Tumor Cell Invasion

Karen E. Hayes

Nearly 40,000 new cases of head and neck squamous cell carcinoma (HNSCC) are annually diagnosed in the United States. The current standard of care for HNSCC patients consists of chemoradiation often combined with targeted therapeutic agents. However, the five-year survival rate for patients that relapse is < 50%. Patients with recurrent or metastatic disease have a very poor prognosis and typical survive < 12 months due to therapeutic resistance and loco-regional invasive spread. HNSCC invasion is mediated in part by lamellipodia and invadopodia, two actin-based structures responsible for facilitating invasive movement. Key signaling pathways that govern lamellipodia and invadopodia production are hyperactivated or overexpressed in HNSCC, including EGFR, Src, Erk 1/2, and cortactin. The overall goal of this dissertation is to determine how these signaling components regulate lamellipodia and invadopodia production and function in HNSCC. Three studies were completed that address these issues. Study One reveals an anti-invasive function for Abl in invadopodia regulation in HNSCC, results that are contrary to the pro-invasive influence Abl has in breast and other cancer types. Study Two establishes a fundamental role for Src has in HNSCC invasion and metastasis through the use of the dual Src/Abl inhibitor saracatinib. In Study Three we determine the expression and activation levels of Erk 1/2 in HNSCC patient samples and the role of Erk 1/2 cortactin phosphorylation in HNSCC adhesion, migration and lamellipodia persistence. Collectively these studies shed new light into the fundamental mechanisms utilized during various steps in HNSCC invasion, providing the potential for refinement and development of new avenues for therapeutic intervention.

## **Acknowledgements**

Well I finally have reached the end of my journey; it has been a long and bumpy road. This is by no means a road that I have travel on my own, throughout my time at West Virginia University I have meet many kind people who have helped me along my way. I would like to take this time to express my gratitude to the people who have enriched my life here at WVU. First, I would like say that I am sincerely gratefully to the graduate school at WVU for giving me this opportunity, I would never been able to obtain a PhD without the financial assistance this program has provided. I would also like to thank my committee members; you have pushed me to become a better research scientist, thank you for your insights and recommendations throughout my graduate career. Next, I wish to express my sincerest gratitude to my mentor, Dr. Scott Weed, his guidance, instruction and patience have allowed me to develop as a scientist. It may have been a struggle but I think that the training wheels can now come off now.

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## Glossary

|                   |  |
|-------------------|--|
| A                 | alanine  |
| Å                 | angstrom   |
| Abl (Abl1, c-Abl) | abelson tyrosine kinase  |
| ABP               | actin binding protein  |
| ACK1              | activated Cdc42-associated kinase                              |
| ADAM              | a disintegrin and metalloproteinase                            |
| ADP               | adenosine diphosphate  |
| AFAP110           | actin filament-associated protein of 110 kDa                   |
| Akt (PKB)         | protein kinase B   |
| AMAP1 (ASAP1)     | AMY-1-binding protein 1  |
| ANOVA             | analysis of variance   |
| Arf6              | ADP-ribosylation factor 6                                      |
| Arg (Abl2)        | Abelson-related gene   |
| Arp2/3            | actin related protein 2/3                                      |
| AREG              | amphiregullin  |
| ATM               | ataxia telangiectasia mutated protein                          |
| ATP               | adenosine triphosphate   |
| BAX               | Bcl-2-associated X protein                                     |
| Bcl-2             | B-cell lymphoma 2  |
| BCR               | breakpoint cluster region                                      |
| BCR-Abl           | breakpoint cluster region- ableson                             |
| bFGF              | basic fibroblast growth factor                                 |
| BPGAP1            | BCH domain-containing Cdc42GAP-like protein                    |
| BSA               | bovine serum albumin   |
| C                 | Celsius  |
| CAFs              | cancer associated fibroblasts                                  |
| CAS               | Crk-associated substrate                                       |
| Cbl               | Casitas B-lineage lymphoma gene                                |
| CBP90             | cortactin-binding protein 90                                   |
| CD2AP             | CD2- associated protein  |
| Cdc42             | cell division cycle 42, GTP binding protein                    |
| CDK2              | cyclin-dependent kinase 2                                      |
| CEF               | chicken embryo fibroblast                                      |
| Cer               | cerulean fluorescent protein                                   |
| CFP               | cyan fluorescent protein                                       |
| CIN85             | Cbl-interacting 85-kDa protein                                 |
| CIP4              | Cdc42-interacting protein                                      |
| c-kit             | cellular homologue of the feline sarcoma viral oncogene v-kit  |
| c-Met             | MNNG HOS transforming gene ; hepatocyte growth factor receptor |
| CML               | chronic myelogenous leukemia                                   |
| CMV               | cytomegalovirus promoter                                       |
| CortBP1           | cortactin binding protein 1                                    |
| CP                | capping protein  |
| Crk I/II          | Cdc2 related kinase 1/2  |

|               |   |
|---------------|---|
| Csk           | C-terminal Src kinase   |
| CTTN          | human cortactin gene  |
| Cttn          | murine cortactin gene   |
| DAB           | diaminobenzidine  |
| DAPI          | 4', 6-diamidino-2-phenylindole                                      |
| DDB1/2        | damaged DNA-binding 1/2   |
| DDW           | arginine-arginine-tryptophan  |
| BK channels   | large conductance calcium- and voltage-activated potassium channels |
| DKO           | double knockout   |
| DMEM          | dulbecco's modified eagle medium                                    |
| DMSO          | dimethyl sulfoxide  |
| DNA           | deoxyribonucleic acid   |
| DNAPK         | DNA-activated protein kinase  |
| DOK           | downstream of tyrosine kinase                                       |
| DRF           | diaphanous-related formin   |
| DUSP          | dual specific phosphatase   |
| E             | epithelial  |
| ECIS          | electric cell-substrate impedance sensing                           |
| ECM           | extracellular matrix  |
| EDTA          | ethylenediaminetetraacetic acid                                     |
| EGF           | epidermal growth factor   |
| EGFR          | epidermal growth factor receptor                                    |
| EMT           | epithelial to mesenchymal transition                                |
| EphR4         | ephrin R4   |
| ERK1/2        | extracellular signal regulated kinase1/2                            |
| Ets           | E-twenty six  |
| F             | filamentous   |
| FAB           | F-actin binding   |
| FACS          | fluorescence-activated cell sorting                                 |
| FAK           | focal adhesion kinase   |
| FBS           | fetal bovine serum  |
| Fer           | feline encephalitis virus-related kinase                            |
| FGD1          | faciogenital dysplasia protein 1                                    |
| FGF           | fibroblast growth factor  |
| FGFR          | fibroblast growth factor receptor                                   |
| FITC          | fluorescein isothiocyanate  |
| FUS1          | fusion 1 protein  |
| Fyn (Syn,Slk) | Fgr/Yes related novel protein                                       |
| G             | globular  |
| GAP           | GTPase-activating protein   |
| GCPR          | g-coupled protein receptors   |
| GEF           | guanine nucleotide exchange factor                                  |
| GFP           | green fluorescent protein   |
| GIST          | gastrointestinal stromal tumor                                      |
| Grb2          | growth factor bound protein 2                                       |

|            |  |
|------------|--|
| GTP        | guanosine-5'- triphosphate                                   |
| HA-tagged  | hemagglutinin tagged   |
| HDAC1      | histone deacetylases 1                                       |
| H & E      | hematoxylin and eosin  |
| HER        | Human Epidermal Growth Factor Receptor                       |
| HGF        | hepatocyte growth factor                                     |
| HIP1R      | Huntingtin-interacting protein1 related                      |
| HP         | helical proline  |
| HNSCC      | head and neck squamous cell carcinoma                        |
| IGFR       | insulin-like growth factor 1 receptor                        |
| IQGAP      | IQ motif containing GTPase-activating protein                |
| IHC        | immunohistochemistry   |
| JAK        | Janus kinase   |
| JNK        | c-Jun N-terminal kinase                                      |
| kDa        | kiloDalton   |
| K-RAS      | Kirsten rat sarcoma viral oncogene homolog                   |
| KSR1       | kinase suppressor of Ras1                                    |
| LSP1       | lymphocyte-specific protein 1                                |
| MAPK       | mitogen-activated protein kinase                             |
| mCh        | mCherry fluorescent protein                                  |
| MDM2       | murine double minute 2                                       |
| MEF        | mouse embryonic fibroblast                                   |
| MEK1/2     | MAP kinase kinase 1/2  |
| MIM        | missing in metastasis  |
| MIBs       | multiplexed inhibitor beads                                  |
| MLCII      | myosin light chain II  |
| MLCK       | myosin light chain kinase                                    |
| MMP        | matrix metalloprotease                                       |
| Mr         | mass relative  |
| MT-MMP     | matrix bound matrix metalloprotease                          |
| MT1-MMP    | matrix bound matrix metalloprotease 1                        |
| mTor       | mammalian target of rapamycin                                |
| MTT        | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADPH      | nicotinamide adenine dinucleotide phosphatase                |
| NCK        | non-catalytic region of tyrosine kinase adaptor protein      |
| NF-κB      | nuclear factor-κB  |
| NI         | not infected   |
| NOXA       | NADPH oxidase activator                                      |
| NPF        | nucleation-promoting factor                                  |
| NSCLC      | non-small cell lung carcinoma                                |
| NTA        | N-terminal acidic domain                                     |
| N-WASp     | Neuronal Wiskott-Aldrich Syndrome Protein                    |
| p120RASGAP | Ras GTPase activating protein, 120 kDa                       |
| p190RhoGAP | Rho GTPase activating protein, 190 kDa                       |
| Pak1       | p21-activated protein kinase                                 |
| PAK3       | p21-activated protein kinase 3                               |



|           |   |
|-----------|---|
| PBS       | phosphate buffered saline                                 |
| pCAF/p300 | P300/ CREB-binding protein-associated factor              |
| PDGF      | platelet-derived growth factor                            |
| PDGFR     | platelet-derived growth factor receptor                   |
| PI3K      | phosphoinositide-3 kinase                                 |
| PIP2      | phosphatidylinositol-4,5-bisphosphate                     |
| PIP3      | phosphatidylinositol 3, 4, 5-triphosphate                 |
| PKC       | protein kinase C  |
| PKD       | protein kinase D  |
| PLC       | phospholipase C   |
| PLCy      | phospholipase-C gamma-1                                   |
| PLL       | poly-L-lysine   |
| PP2A      | protein phosphatase 2A                                    |
| PP2C      | protein phosphatase 2C                                    |
| PRR       | proline-rich region                                       |
| PSS       | phosphate saline solution                                 |
| PTB       | phosphotyrosine binding domain                            |
| PTPa      | protein tyrosine phosphatase a                            |
| PTP1B     | protein tyrosine phosphatase 1B                           |
| PTPPEST   | protein tyrosine phosphatase-PEST                         |
| PTPN1     | protein tyrosine phosphatase, nonreceptor-type 1          |
| PUMA      | p53 upregulated modulator of apoptosis                    |
| Pyk2      | proline-rich tyrosine kinase 2                            |
| R         | arginine  |
| Rab7/8    | Ras-associated binding 7/8                                |
| Rac       | Ras-related C3 botulinum toxin substrate 1                |
| RAF       | Rapidly Accelerated Fibrosarcoma oncogene                 |
| Rb        | retinoblastoma protein                                    |
| RhoA      | Ras homolog family member A                               |
| RhoGAP    | Rho family GTPase-activating protein                      |
| RIPA      | radioimmunoprecipitation assay                            |
| RNAi      | RNA interference  |
| ROCK      | Rho-associated serine/threonine kinase                    |
| ROIs      | reactive oxygen intermediates                             |
| ROS       | reactive oxygen species                                   |
| RSV       | Rous sarcoma virus  |
| RTK       | receptor tyrosine kinase                                  |
| S         | serine  |
| SDS-PAGE  | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEF       | similar expression to fgf genes                           |
| SFK       | Src family kinases  |
| SH1       | tyrosine kinase   |
| SH2       | Src homology 2  |
| SH3       | Src homology 3  |
| SH4       | Src homology 4  |
| SHANK     | SH3/ankyrin domain gene                                   |

|               |   |
|---------------|---|
| Shc           | Src homology 2 containing                                       |
| SHP-2         | Src homology 2-containing tyrosine phosphatase                  |
| SiRNA         | small interfering ribonucleic acid                              |
| SIRT1         | silent mating type information regulation 2 homolog 1 (sirtuin) |
| SNARE         | soluble NSF attachment protein receptors                        |
| SOS           | son of sevenless  |
| Src           | sarcoma kinase  |
| STAT          | signal transducer and activator of transcription                |
| SYF           | Src-/-Yes-/-Fyn-/-  |
| SYF+/+        | Src+/+ Yes-/-Fyn-/-   |
| Syk           | spleen tyrosine kinase  |
| Tg            | transgenic  |
| TGF $\alpha$  | transforming growth factor $\alpha$                             |
| TGF $\beta$   | transforming growth factor $\beta$                              |
| Tks5          | five SH3 domain (fish)/SH3 and PX domains protein 2A            |
| TPM           | triple point mutant   |
| TRITC         | tetramethyl rhodamine iso-thiocyanate                           |
| Trp53         | transformation related protein 53 gene                          |
| <i>tsLa29</i> | temperature-sensitive viral Src clone 29                        |
| TYM           | triple tyrosine mutant  |
| uPAR          | urokinase type plasminogen activator                            |
| vAbl          | viral Ableson   |
| VASP          | vasodilator-stimulated phosphoprotein                           |
| VDAC          | voltage-dependent anion channel                                 |
| VEGF          | vascular endothelial growth factor                              |
| v-Src         | viral sarcoma gene  |
| W             | tryptophan  |
| WASp          | Wiscott Aldrich Syndrome protein                                |
| WAVE          | WASp family verprolin-homologous protein                        |
| WH2           | WASp homology domain  |
| WIP           | WASp interacting protein  |
| WT            | wild-type   |
| Y             | tyrosine  |
| ZO-1          | Zonula occludens protein 1                                      |

## **Literature Review**

### **Head and neck squamous cell carcinoma.**

Head and neck squamous cell carcinoma (HNSCC) arises in the mucosal epithelial linings of the oral cavity, oropharynx, nasopharynx, larynx and hypopharynx (1,2). In the United States approximately 40,000 patients are diagnosed with HNSCC each year, comprising 3% to 5% of all cancer patients (3). HNSCC can be classified as human papilloma virus (HPV)-positive or HPV-negative. The major risk factors of HPV-negative HNSCC are sustained tobacco and alcohol use, with 90% of HNSCC cases having a positive correlation. Recently tobacco-associated HNSCC diagnoses have declined, likely due to the decrease in tobacco consumption in the United States over the last eleven years. Conversely, the number of HPV-positive HNSCC patients continues to increase. (3-5).

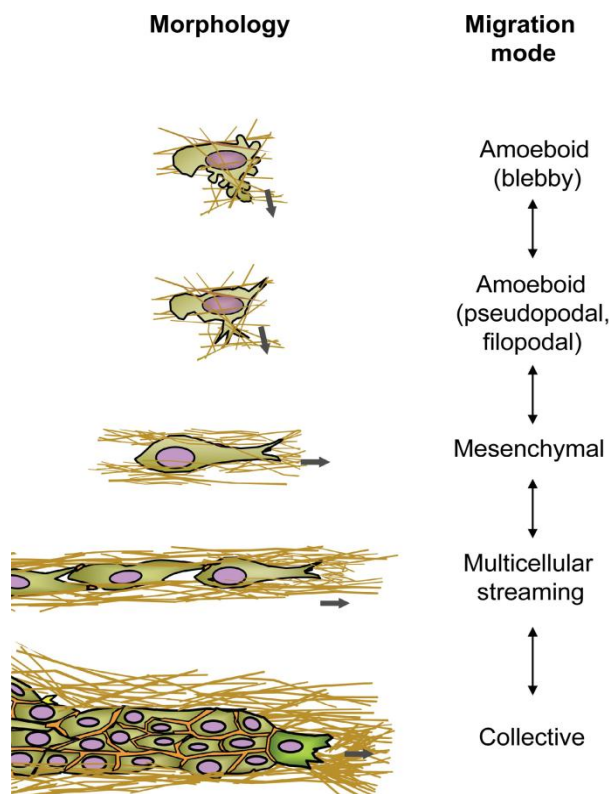
Presently, HPV-positive (predominantly HPV type-16) HNSCC patients comprise approximately 20% of all HNSCC cases, of which 60% to 80% are oropharyngeal-based cancers (5). HPV is a small circular double stranded DNA virus encoding sequences for early proteins E1, E2 and E4-E7 and late proteins L1 and L2. HPV transforms epithelial cells via E6- and E7-mediated regulation of tumor suppressors, p53 and Rb and other key components while E5 modulates epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) internalization (5,6). In general, HPV-positive HNSCC primary tumors are smaller, poorly differentiated and have a 5-year survival rate between 70-80%. This survival rate is significantly better than the 5-year rate for HPV-negative HNSCC, which is less than 50%. Both HNSCC sub-types receive the same standard treatment consisting of tumor resection, post-operative irradiation therapy and/or platinum-based chemotherapy (3,4,7). Regardless of HPV designation, recurrent and/or metastatic HNSCC patients have an abysmally poor prognosis with a median survival rate of less than one year despite the addition of concomitant treatment with the EGFR-targeted pharmacological agent cetuximab (4,8).

In general HNSCC presents as a locoregional disease involving metastasis to cervical lymph nodes, but distant metastases arise occasionally in lungs, bone, or liver of

HNSCC patients (9). Metastasis is a multi-faceted processes, involving invasion through the basement membrane and extracellular matrix (ECM), intravasation and extravasion through vascular endothelial layers, and colonization at distant sites (9,10). The heterogeneity of tumors and the complexity of the microenvironment perpetuate a multitude of proposed modes of cell invasion, from collective to single cell amoeboid/mesenchymal phenotypes (Figure 1) (11).

### Methods of cancer cell motility and invasion.

Amoeboid invasion (blebbing or pseudopodal) is generated though physical force that permits rounded, poorly attached individual tumor cells to push through gaps in loose connective tissue and induce non-proteolytic deformation of the extracellular matrix. Amoeboid invasion utilizes bleb-like membrane protrusions, pseudopodia (Rac GTPase induced cylindrical actin-network based membrane protrusions), or filopodia (Cdc42 GTPase mediated slender membrane protrusions containing cross-linked parallel filamentous (F)-actin bundles at the leading edge of polarized motile cells. These structures assist in mediating the speed and trajectory of the cell during amoeboid



**Figure 1** Plasticity of tumor cell migration. Modes of tumor cell migration are classified by cellular morphology and proteolytic activity. Individual cell movements: amoeboid mediated by protease independent and contractile forces pushing through loose connective tissue, or mesenchymal mediated by protease-dependent degradation of extracellular matrix. Collective cell movements: multicellular streaming mediated by chemotactic signaling and pathways generated by matrix remodeling, or collective movement reliant focalized ECM degradation and cell-to-cell contacts to generate directionality.

Friedl, P. and Wolf, K. (2010) *J. Cell. Biol.*, **188**(1), 11-19 (modified).

movement (11-13). The myosin II influenced contractile forces utilized to push the cell forward are generated via Rho-mediated activation of the Rho-associated serine/threonine kinase (ROCK), resulting in simultaneous inactivating phosphorylation of myosin phosphatase and subsequent persistent phosphorylation of myosin light chain on threonine 18 and serine 19 to collectively drive cell contraction (14,15).

Mesenchymal motility is a multi-step process dependent on proteolytic activity to remodel connective tissue. Spindle shaped cells in response to spatial and temporal signaling extend actin-based protrusion classified as invadopodia. Invadopodia are proteolytic actin-rich structures that degrade the ECM or basement membrane. Invadopodia-generated motility occurs via actomyosin-mediated contraction that propels the cell forward along with retraction of cell posterior (16,17). The plasticity of tumor cells allows transition from amoeboid and mesenchymal invasion (and vice versa) based on microenvironment conditions as well as intra- and extracellular signaling cues (11,18).

Collective cell movement encompasses invading cellular streams and cellular sheets that maintain cell-cell contact with each other and possess focalized degradation of ECM at the invasive front. Cellular stream invasion utilizes cancer associated fibroblasts (CAF) or mesenchymal tumor cells to generate microtracks in connective tissue, permitting chain-like migration (19). The intercellular signaling generated from the cell-cell contact directs cellular collective movement (20). Collective invasion is the primary invasion mode utilized by HNSCC (18).

This dissertation focuses on two actin-generated organelles, lamellipodia and invadopodia, that are necessary for these modes of invasion. Lamellipodial-based 2D invasion occurs at the leading edge of motile cells and possess little to no proteolytic activity. Lamellipodia are dynamic structures that last only minutes in vitro; they are responsible for facilitating rapid directional movement. Conversely, invadopodial-based 2D invasion is mediated by proteolytic degradation of ECM on the ventral surface of the cell. Invasion occurring via invadopodia is generally slow, with invadopodia structures lasting up to several hours (21). Lamellipodia (or pseudopodia; the 3D equivalent) are thin sheet-like membrane protrusions induced by extrinsic chemokines or adhesion

signaling that function by attaching to the ECM and pulling the cell forward. The prototypical Rho-family GTPases Rac1, RhoA and Cdc42, as well as receptor tyrosine kinases and phospholipids propagate cytoplasmic signaling responsible for stimulating branched actin-based cytoskeletal networks generating the force required to extend the membrane (22,23). Actin nucleating promoting factors (NPFs) are responsible for lamellipodia extension, where the actin-related protein 2/3 (Arp 2/3) complex forms branched actin networks, while NPFs of the formin family (e.g.; mDia1), and Spire (Cordon Blue) facilitate straight actin filament formation that serves as the basis for subsequent branched Arp2/3-produced networks (24,25).

First observed by Chen et. al., invadopodia are protrusive invasive structures that have focalized matrix proteolytic activity (26,27). Invadopodia utilize many of the same regulatory and actin-nucleating proteins observed in lamellipodia. However, invadopodia also require the components needed to form microtubules and secrete matrix metalloproteases to facilitate ECM degradation. This collective activity allows resulting membrane protrusions to penetrate the basement membrane in order to remodel ECM (28-30). Invadopodia are complex structures comprising of a central Arp 2/3-generated F-actin core. Key actin polymerization regulators, including Arp 2/3, cortactin, Tks 5 and N-WASp localize to the core region and are required for invadopodia formation (30-34).

Invadopodia are produced following stimulation by extracellular cues, where Ras family GTPases, such as CDC42/CDC42 GEF (Fgd1), non-receptor and receptor tyrosine kinases (RTKs) are activated (18,21,32,35-38). Src-phosphorylated Tsk5 binds and recruits the adaptor proteins Nck, cortactin and N-WASp to pre-invadopodia sites (34,39,40). WIP, Nck, and N-WASp bind Src or Abl/Arg tyrosine kinases, which in turn tyrosine-phosphorylate cortactin to create docking sites for multimeric protein complexes (see below for details) that amplify Arp 2/3-mediated actin branching and subsequent invadopodial membrane protrusion (30,36,37,41). Additional actin binding proteins assist in modulating invadopodia activity. For example, the actin binding protein cofilin regulates invadopodia assembly/disassembly by inducing barbed-end generation, Arp2/3 filament debranching and actin turnover (32,37,42). Microtubules and intermediate filaments extend into nascent invadopodia and are essential for

elongation (43). Invadopodia also contain radial, unbranched, bundled actin filaments that are polymerized by formins, where filament ends are uncapped by Ena/Vasp proteins to facilitate direct actin filament elongation by allowing preferential monomer addition at the “plus” end near the invadopodia tip (43-46). Parallel actin filaments are bundled by the proteins fascin,  $\alpha$ -actinin and caldesmon to provide structural integrity to the actin core (43,47-49). The adhesion proteins talin and paxillin localize to invadopodia and mediate integrin-based contact with ECM, but oddly the adhesion proteins focal adhesion kinase (FAK) and vinculin are not found in invadopodia (49-51). On a cellular level, adhesion and ECM degradation are inversely but tightly regulated; where degradation of matrix at sites of adhesion is counterproductive to adhesion-based force generation (16,18,52).

Focalized ECM degradation occurs at tips and branch points of elongated invadopodia (16,18,52,53). Several proteinases localize to invadopodia that are responsible for facilitating ECM degradation. These include matrix metalloproteinases MT1-MMP (MMP14), MMP2, MMP9, ADAM-family sheddases, cathepsin, seprase, and urokinase type plasminogen activator (uPAR)) (21,29,54-59). MT1-MMP is widely considered the major and essential zinc-dependent membrane-bound MMP in regulating and enabling invadopodia proteolytic activity (the last stage in functional invadopodia maturation), as manipulation of MT1-MMP expression or activity directly modulates matrix degradation and invasion (29,57,60-62). MT1-MMP localization to invadopodia can occur through clatherin-mediated endocytosis (63-66) or by exocytic membrane trafficking (67-69).

Although the majority of work imaging invadopodia has been performed in 2D settings, invadopodia are believed to exist *in vivo*, since invadopodia forming cancer cell lines are more invasive in xenografts models than lines that do not form invadopodia. Also, primary cells from several tumor types, including HNSCC, bladder cancer and glioblastomas form functional invadopodia in classic 2D gelatin matrix degradation assays (70-73). In addition, invadopodia-like structures have been imaged in physiological relevant *in vivo* and *ex vivo* microenvironments containing key proteins associated with invadopodia, including N-WASp, Tks5, cortactin and Arp 2/3 that result in proteolysis of 3D ECM (69,74-77). Finally, actin-rich protrusive degradative

structures termed podosomes are related to invadopodia, but are found in non-transformed cell types where they are essential for homeostatic invasive processes conducted by osteoclasts, macrophages, smooth muscle and endothelial cells (21,78).

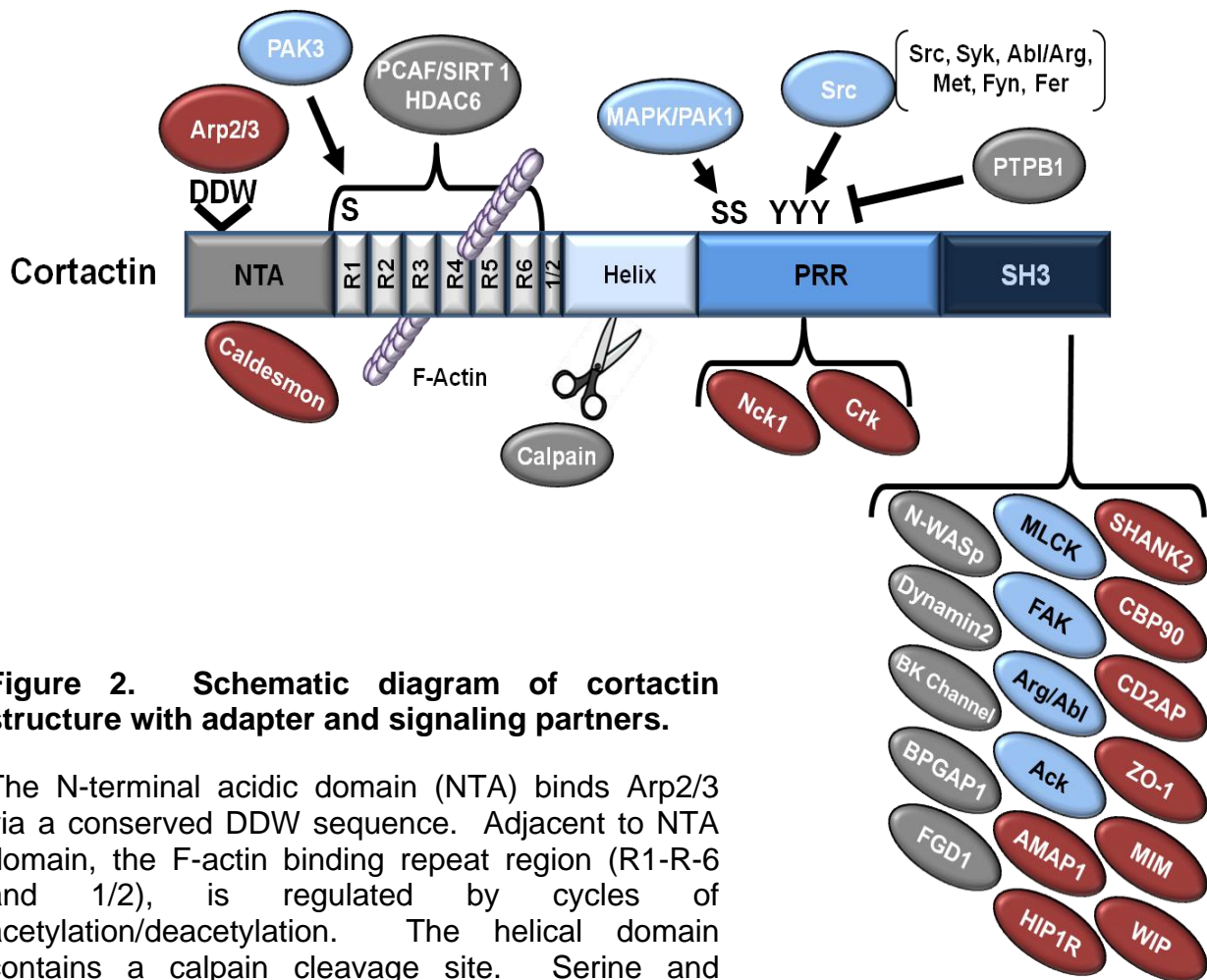
### **Cortactin: A node of actin network regulation.**

Wu and Parsons identified cortactin as a substrate of Src kinase that localizes to cortical F-actin over 20 years ago (79). Cortactin is a NPF that simultaneously activates Arp 2/3 actin polymerization and also serves to stabilize resultant branched actin networks in lamellipodia and invadopodia (80). Cortactin is overexpressed in several different cancer types, including HNSCC (30% of cases), breast (15% of cases), lung, and bladder. Notably, cortactin overexpression in these and other tumor types is primarily due to amplification of the cortactin (CTTN) gene on chromosome 11q13 (81-86), where cortactin gene amplification has emerged as a prognostic marker for poor outcome, corresponding with increased risk for tumor recurrence and lymph node metastasis (84,87-90).

Cortactin is a multi-domain, rod-shaped protein that folds back onto itself to form a “lollipop” shaped structure containing a globular region produced via intramolecular interactions (80,91). Cortactin consists of five domains: an amino-terminal acidic domain (NTA), an F-actin binding domain (FAB), a helical region, a proline-rich region (PRR), and a Src-homology 3 domain (SH3) (Figure 2) (92). The NTA region of cortactin binds Arp 2/3 via the highly conserved DDW amino acid sequence (amino acids 20-22) found in other NPF proteins (93-96) that directly activates in vitro Arp 2/3 nucleation activity (97). Arp 2/3 activation by cortactin is enhanced through additional indirect mechanisms that include the recruitment of cortactin binding proteins to other cortactin domains (41,98-100). Adjacent to the NTA region, the F-actin binding domain consists of six and a half 37 amino tandem repeats that bind directly bind F-actin (79,101). F-actin binding is regulated by acetylation/deacetylation of lysine residues within the repeats region. The histone acetyltransferases pCAF/p300 and SIRT1 acetylate cortactin and prevent F-actin binding, whereas the histone deacetylase HDAC6 deacetylates cortactin to facilitate F-actin re-binding (102-104). Cortactin binding to F-actin results in increased cell migration and is necessary for invadopodia



formation and function (104-107). The helical region contains a calpain cleavage site that results in impaired lamellipodia protrusion and increased migration when proteolyzed by calpain 2 (108). The PRR and SH3 domains constitute regions that are responsible for receiving upstream signaling inputs via phosphorylation and regulated interaction of SH3 binding partners (Table 1) that ultimately govern downstream actin-mediated cellular processes (21,80).



**Figure 2. Schematic diagram of cortactin structure with adapter and signaling partners.**

The N-terminal acidic domain (NTA) binds Arp2/3 via a conserved DDW sequence. Adjacent to NTA domain, the F-actin binding repeat region (R1-R6 and 1/2), is regulated by cycles of acetylation/deacetylation. The helical domain contains a calpain cleavage site. Serine and tyrosine residues in the proline-rich region (PRR) are phosphorylated by Src family and MAPK kinases, creating docking sites for SH2 domain proteins or mediating conformational changes to allow ligand binding to the SH3 domain. The carboxyl-terminal SH3 domain binds several proline-rich proteins regulating multiple diverse cellular processes. *Adaption from L. Kelley dissertation, 2010.*

**Table 1. Table of cortactin SH3 binding proteins.**

| <b>Cortactin SH3 Binding Protein</b> | <b>Function</b>   | <b>Reference</b>        |
|--------------------------------------|---|-------------------------|
| Abl/Arg                              | Phosphorylation of Y421, Y470, Y486 regulating dorsal waves, lamellipodia and invadopodia   | (110,111,310)           |
| CBP90                                | A “brain specific” protein that bind cortactin at synaptosomal membrane, function unknown   | (377)                   |
| ZO-1                                 | Tight junction adaptor, binds cortactin regulating cell adhesion and spreading  | (378-380)               |
| BPGAP1                               | RhoA-GAP, facilitates cortactin translocation to cell periphery increasing cell migration   | (381,382)               |
| HIP1R                                | Caps barbed ends inhibiting actin polymerization mediating clatherin-mediated endocytosis   | (383)                   |
| BK channels                          | large conductance calcium- and voltage-activated potassium ion channels, cortactin mediates Src regulation of BK channel function | (384,385)               |
| ASAP1/AMAP1                          | Recycling endosomes focal adhesions, invadopodia podosomes/ ARF6 GAP  | (386-390)               |
| N-WASp                               | Neucleating promoting factor, enhances cortactin-based Arp2/3 nucleation activity   | (32,37,100,121,391-393) |
| Dynamin 2                            | GTPase, cortactin links dynamin2 and actin filament facilitating dynamin2 remodeling of actin filaments, membrane trafficking     | (277,394-397)           |
| CortBP1/SHANK                        | Scaffold protein in postsynaptic sites, binds cortactin modulating actin remodeling   | (398-400)               |
| FGD1                                 | CDC42-GEF, enhances cortactin mediated Arp2/3 actin polymerization  | (401,402)               |

|                                  |  |              |
|----------------------------------|--|--------------|
| WIP                              | Forms complex with N-WASp and cortactin enhancing Arp2/3 mediated actin polymerization   | (98,100,403) |
| Myosin Light Chain Kinase (MLCK) | Cortactin/MLCK interaction enhances myosin light chain phosphorylation and positively regulates endothelial cell barriers  | (404-407)    |
| Missing in Metastasis (MIM)      | Enhances cortactin mediated Arp 2/3 actin polymerization increasing cortactin induce motility. Src-mediated phosphorylation of cortactin is inhibited by MIM, enhancing cilia formation  | (408-410)    |
| CD2AP                            | Cortactin binds/recruits CD2AP to lamellipodia, CD2AP recruits barbed end capping protein (CP) to lamellipodia. CD2AP binds cortactin and EGFR regulating EGFR endocytosis               | (411,412)    |
| ACK                              | ACK1 phosphorylates cortactin; ACK1/cortactin interaction mediates EGFR internalization  | (413)        |
| FAK                              | Non-receptor tyrosine kinase, FAK/Src complex binds and phosphorylates cortactin stimulating focal adhesion turnover and cell motility and regulates irradiation induced JAK activation. | (133-135)    |

The cortactin PRR contains three key tyrosine residues (Y421, Y470, and Y486 in humans) that are phosphorylated by the Src family kinases Src, Fyn, Syk, and Fer, as well as the Abl family kinases Abl and Arg (109-113). Phosphorylation at these residues creating binding sites for the Src homology 2 (SH2) domain containing adaptors proteins Nck1 and Crk, as well as mediating Abl family kinase binding (100,110,111,114). In the case of Nck1, the Nck1 SH2 domain binds pY421 and pY466, and recruits the Arp 2/3 NPF, N-WASp; N-WASp PRR domain binds cortactin SH3 domain creating a ternary complex that amplifies cortactin-based Arp2/3 nucleation activity indirectly through promoting N-WASp activation (41,100). The net result of cortactin tyrosine phosphorylation is increased invasion and metastasis by enhancing lamellipodia persistence, dorsal wave formation and invadopodia ECM degradation activity (37,41,110,111,115-119). Protein tyrosine phosphatase 1B (PTP1B) interacts

with and dephosphorylates cortactin, consequently reducing cortactin-mediated invasion and metastasis (120).

Extracellular signal-regulated kinases Erk 1/2 (MAPK 3/1), are two related serine/threonine kinases that phosphorylate S405 and S418 in the cortactin PPR. Erk phosphorylation causes a characteristic shift in the *Mr* of cortactin from 80 to 85 kDa in SDS-PAGE due to conformational alterations rather than simple addition of phosphate moieties (121-123). The close proximity of the targeted serine and tyrosine phosphorylation sites (especially S418 and Y421) has led to proposals where interplay between these two different phosphorylation events function in tandem to regulate cortactin signaling inputs. Work by Martinez-Quilles et al. (2004) with various tyrosine and serine cortactin phosphorylation mutants in *in vitro* biochemical assays indicated that serine phosphorylation by Erk 1/2 enhanced cortactin SH3 domain binding to the N-WASp, PRR, resulting in N-WASp activation and Arp 2/3 activation apart from the tyrosine phosphorylation Nck1/N-WASp mechanism described above. Phosphorylation by Src led to decreased N-WASp binding and reduced Arp 2/3 actin polymerization. This group hypothesized that phosphorylation of cortactin serves as an on/off switch, where serine phosphorylation disrupts the intra-molecular interactions holding cortactin in a globular form to allow proline-rich proteins (e.g. N-WASp) to bind the exposed SH3 domain and become activated (the so-called “active” cortactin form). Conversely, tyrosine phosphorylation was proposed to promote the closed conformation by permitting re-folding of cortactin into the globular “inactive” conformation. While the precise mechanism of how Src-based tyrosine phosphorylation promotes cortactin inactivation was unclear, it was proposed that the modes of the serine/tyrosine switch function independently (121). Our lab (Study 3) demonstrated through the use of phosphospecific antibodies against cortactin Y421 and S418 that phosphorylation of serine and tyrosine residues occur simultaneously in cells, in contrast to these earlier findings (123,124). Collectively these results suggest that cortactin tyrosine phosphorylation does not confer a closed “inactive” conformation proposed by the “serine-tyrosine switch”.

In addition to Src-, Abl- and Erk-family kinases, cortactin is also targeted by a variety of other kinases. The serine/threonine kinase p21-associated kinase (PAK1) phosphorylates S405/S418 following activation of Rac1 to regulate N-WASp-mediated vesicle motility (125). Phosphorylation of cortactin S113 by PAK3 in the first tandem repeat regulates cortactin binding to F-actin (126). Protein kinase D (PKD) targets cortactin at S298/S348, inhibiting WAVE2/Arp2/3 mediated actin polymerization (127-129). In addition to these examples, there are 15 additional cortactin phosphorylation sites identified by mass spectroscopy that currently have unknown functions (130). Moreover, integrin-mediated FAK modulation of cortactin activity has divergent effects in Src-dependent and Src-independent manner. The FAK/Src complex binds and phosphorylates cortactin on Y421, Y470, and Y486 increasing focal adhesion turnover and cell motility (131-134). Integrin engagement also confers radiation resistance via FAK/cortactin downregulation of JNK in a Src-independent manner (135).

The carboxyl-terminal cortactin SH3 domain binds a multitude of different proteins (Table 1). These binding partners mediate cortactin regulation of various actin-based processes including cellular signaling (Fgd1, BPGAP1, Abl family kinases), actin polymerization (N-WASp, WASp-interacting protein (WIP)), membrane deformation (missing in metastasis, (MIM)), cell-cell and cell-substrate adhesion (ZO1 and FAK), endocytic and exocytic membrane trafficking (ACK1, HIP1R, AMAP, dynamin2, CortBP1/SHANK2, CD2AP), and actomyosin-based contraction (myosin light chain kinase (MLCK) (80,136-138). These processes are collectively involved in cell migration and utilized in the organization invadopodia assembly. Several studies using deletion mutants have clearly determined that the cortactin SH3 domain is essential for proper invadopodia formation and function in cancer cell lines (37,125,139). These results suggest cortactin creates a nexus for invadopodia regulation and subsequently serves as a driver of cancer cell invasion.

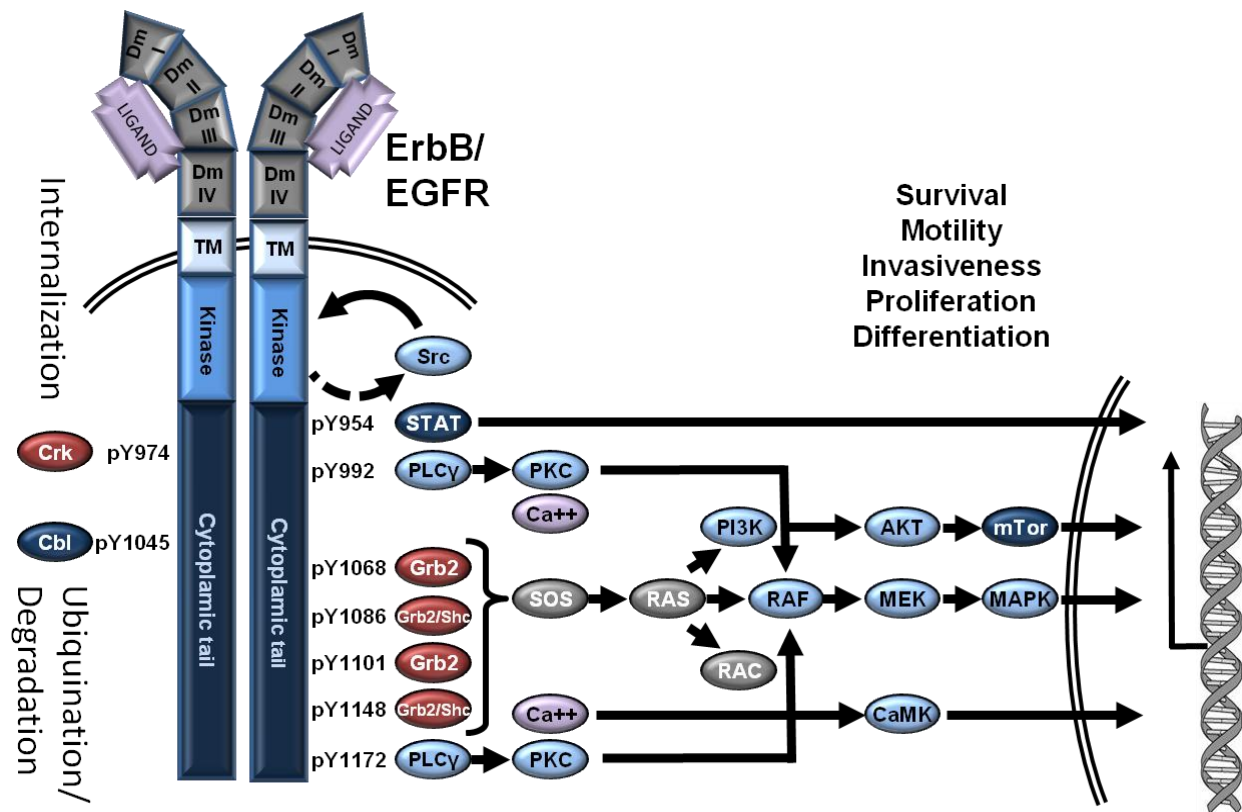
### **EGFR: Signaling and significance in HNSCC.**

Epidermal growth factor receptor (EGFR, HER1, ErbB1) is a transmembrane tyrosine kinase that is a member of the ErbB family. Other members include ErbB2 (HER2), ErbB3, and ErbB4 (140). EGFR contains an N-terminal extracellular ligand binding

region (domains I-IV), a hydrophobic transmembrane region, and a C-terminal cytoplasmic consisting of tyrosine kinase and autophosphorylation regions that propagate “outside-in” signaling (Figure 3) (140,141). There are thirteen distinct ligands that can bind one or more ErbB receptors, adding to the complexity of ErbB signaling (142,143). ErbB ligands are produced in the “pro” form and then transported to the plasma membrane, where they are inserted into the membrane or are exocytosed. In HNSCC, the sheddases ADAMs 10 or 17 cleave the pro-ligand to create an autocrine or paracrine signal (144,145). Alternatively, the uncleaved EGFR ligand heparin binding-epidermal growth factor (HB-EGF) can bind adjacent cells to generate a juxtacrine signal (146). Epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF $\alpha$ ) and amphiregullin (AREG) are common ErbB ligands that preferentially bind EGFR and are overexpressed in HNSCC, although other EGFR ligands also play lesser roles in HNSCC progression (143,147,148).

Following ligand binding, ErbB receptors undergo a conformational change exposing a “dimerization arm” in domain II of the receptor to permit hetero- or homo-dimerization with a second ErbB receptor. Dimerization activates the tyrosine kinase domain, facilitating cross autophosphorylation of ten tyrosine sites of dimerized ErbB receptor cytoplasmic tail. The phosphorylation pattern varies depending on the ErbB/ligand combination (149,150). Phosphorylated tyrosines in the EGFR cytoplasmic region bind SH2 domain-containing scaffold proteins (e.g. Shc, Crk, and Grb2) as well as downstream signaling proteins (e.g. STAT, PLC $\gamma$ , and Cbl). EGFR activation regulates several critical signaling pathways, mainly JAK2/STAT, RAS/Raf/MAPK, and PLC $\gamma$ /PI3K/Akt to govern cellular processes that include proliferation, survival, and motility (Figure 3) (151-155). EGFR signal attenuation occurs via clathrin-coated mediated endocytosis, with the targeting of EGFR-containing endosomes for surface recycling, degradation, or nuclear localization based on affinity of ligand and phosphorylation status. ErbB receptors with weakly bound ligands (e.g.; EGFR/TGF $\alpha$ ) are recycled from early endosomes back to cell surface, whereas highly phosphorylated ErbB receptors are targeted for lysosomal degradation via late endosomes. Termination of EGFR signaling is assured through SH2 domain-mediated binding of the E3 ubiquitinase Cbl to phosphorylated tyrosine 1045 on EGFR, leading to ubiquitination

of EGFR to target it for proteasome-mediated degradation (156-159). EGFR is also targeted to the nucleus through importin  $\beta$  retrograde transport (160,161), where it regulates cell proliferation and DNA repair. Nuclear EGFR is also an indicator of poor prognosis in cancer patients (162,163).



**Figure 3. Schematic diagram of EGFR and conical MAPK signaling pathways.**

Summary of EGFR-based signal transduction. EGFR extracellular region (domains (dm) I-IV) binds ligand, dimerizes and cross-phosphorylates a second ErbB receptor. Activated EGFR triggers three MAPK pathways: JAK/Stat, PI3K/Akt/mTOR, and RAS/RAF/Erk1/2 signaling cascades regulating transcription of genes that modulate cellular processes that induce tumorigenesis. Clathrin-mediated EGFR internalization is regulated by adaptor protein Crk. E3 ubiquitin ligase Cbl targets EGFR for degradation.

In addition to elevated proliferation and survival, activated EGFR regulates cell motility and invasion in HNSCC and other cancer types. EGFR activation stimulates activation of Src and Abl family tyrosine kinases that in turn govern the downstream production of

lamellipodia and invadopodia (36,37,100,111,115,116,164-168). Invasion and motility in HNSCC are directly inhibited with the small molecule EGFR targeting drug gefitinib (115,169,170). EGFR activation in HNSCC also enhances MMP9 expression and activation (171,172). These studies suggest a correlation between EGFR activity and pro-metastatic behavior in HNSCC.

The majority of stage III and IV HNSCC patients develop locoregional recurrences and/or distant metastasis (4,141). EGFR overexpression occurs in  $\geq 90\%$  of all HPV-negative HNSCC, where it correlates with radiation resistant tumors and locoregional failures (170,173). Up-regulation of EGFR signaling in HNSCC is further enhanced by the expression of EGFR ligands. EGF is overexpressed in 65% of cases, TGF $\alpha$  in 90% and AREG in 45% ((170,174,175). Recent work indicates that EGFR expression and activation levels are independent predictors of poor prognosis for HNSCC patients (176). The observed high rate of elevated EGFR activation is likely not due to activating point mutations, which rarely arise in HNSCC. However, a truncated version of EGFR (EGFRVIII) with deletion of exons 2-7 occurs in 17 - 42% of the HNSCC cases (177-179). Exon 2-7 deletion removes the extracellular domain, rendering it constitutively active and resistant to monoclonal antibody-based therapeutic inhibition (177,180,181). In addition to HNSCC, several other solid tumor types overexpress EGFR, including non-small cell lung carcinoma (NSCLC, 62%), breast cancer (50-70%) and glioblastomas (>50%) (182-184).

Multiple therapeutic EGFR inhibitors exist today designed to disrupt or ablate EGFR dimerization or kinase activity. Prominent examples include cetuximab, panitumumab, gefitinib, vandetanib, erlotinib and lapatinib. EGFR targeted therapy can be subdivided into two categories; monoclonal antibodies or small molecule inhibitors (181,185). Cetuximab is a monoclonal antibody that has been approved for concomitant therapy with radiation or platinum-based chemotherapy for recurrent or metastatic HNSCC based on the increased response rate in phase III trial (8,141,186,187). Gefitinib is a small molecular EGFR inhibitor approved for patient treatment. However, combination therapy of gefitinib and methotrexate in phase III trial of chemoresistant recurrent/metastatic HNSCC patients did not improve overall response rate



(181,188,189). The high percentage of different solid tumor types combined with sporadic success of targeted therapeutic approaches continues to keep EGFR in the spotlight as an important molecular target in head and neck oncology.

### **Erk1/2: Effector proteins for EGFR signaling.**

Extracellular signal-regulated kinase 1 and 2 (Erk 1/2, MAPK 3/1) were the first discovered members of the mitogen-activated protein kinases (MAPK) kinase family and are ubiquitously expressed. Erk 1/2 and other MAPK members including c-Jun N-terminal kinase (JNK), p38 MAPK, and Erk5 are serine/threonine kinases activated by various extracellular cues (190,191). Erk 1/2 proteins are comprised of an N-terminal domain that facilitates binding to ATP and a C-terminal catalytic domain containing a conserved “TXY” amino acid sequence targeted by upstream kinases. A channel generated between the two terminal domains creates a binding pocket for Erk 1/2 substrates (192-194).

Erk 1/2 has approximately 180 substrates that impact proliferation, differentiation, cellular survival/apoptosis, differentiation, motility, and invasion (195,196). Given the complexity of Erk 1/2 signaling network, Erk activity is tightly regulated in a spatial and temporal manner. The subcellular localization of Erk proteins is determined by several factors, including binding of Erk 1/2 to the tethering proteins VDAC, SEF, or LSP1 at mitochondria, Golgi, or the plasma membrane, respectively (195). Erk 1/2 compartmentalization is also influenced by the duration and strength of upstream EGFR signals, where strongly EGF-activated EGFR generates a transient cytoplasmic signal, while weakly bound EGFR ligands initiate sustained Erk activation and relocation of Erk kinases to the nucleus (140,195).

The mechanism of EGFR-mediated Erk 1/2 activation is well described. Following EGFR activation, Src homology 2 containing (Shc) protein binds to phosphorylated tyrosines on the EGFR cytoplasmic domain. This in turn recruits growth factor bound protein 2 (Grb2) and son of sevenless (SOS), a RAS guanine nucleotide exchange factor (GEF). SOS activates one of three different RAS isoforms (H-RAS, N-RAS, or K-RAS) by converting GDP-bound RAS to GTP-RAS (197). RAF (A-RAF, B-RAF, or C-

RAF) are a class serine/threonine kinases that localize to the plasma membrane and are activated by GTP-RAS. Activated RAF in turn phosphorylates mitogen-activated protein kinase kinase (MEK or MAP2K), resulting in MEK activation (191,195,198,199). The RAF/MEK complex is stabilized by the scaffold protein kinase suppressor of Ras 1 (KSR1), which also recruits Erk 1/2 (200,201). Activated MEK in turn phosphorylates Erk 1/2 on T183 and T185 to generate an active kinase (202,203). Once activated, the protein complex disassociates and releases activate Erk 1/2, which either localizes to the nucleus to regulate transcription, or to specific cytoplasmic compartments to regulate other cellular processes (detailed in Figure 4) (191,195,198,199,204).

Since Erk 1/2 controls an array of cellular processes, its activation cycle is tightly regulated. Erk 1/2 is inactivated by MAPK phosphatases (PP2A and PP2C) (205) and dual specific phosphatase (DUSP) (206). Erk 1/2 itself also regulates several of the factors involved in the RAS activation pathway. SOS phosphorylation by Erk 1/2 disrupts the SOS/Grb2 interaction (207), Erk1/2-mediated phosphorylation of EGFR Y669 increases EGFR phosphorylation and inhibits EGFR degradation, enhancing EGFR activity (208) RAF and MEK are also inhibited by Erk 1/2 phosphorylation (209,209,210). In addition, DUSP transcription is induced by Erk 1/2 phosphorylation of transcription factor Ets (211). Collectively, Erk 1/2 activation regulates several feedback loops that converge at multiple levels to provide fine tuning of the Erk 1/2 signaling cascade (191,212,213).

In addition to EGFR, Erk 1/2 activation is facilitated by integrin signaling in a non-canonical manner. Integrin-mediated activation of FAK induces activation of Src, which initiates Ras activation to increase Erk 1/2 activity (214-216). Erk activation stimulated by integrin engagement imparts multiple effects that manifest in increased invasion and metastasis in many tumor types. HNSCC cells plated on fibronectin enhances Erk 1/2 activation (217). FAK activity induced by  $\alpha V\beta 3$  integrin engagement enhances Erk and Akt activation, increasing the aggressiveness of chronic myelogenous leukemia (CML) (218). The aforementioned Erk1/2-mediated serine phosphorylation of cortactin regulates invadopodia proteolytic activity in HNSCC and melanoma cell lines (115,123,139) and lamellipodia formation in breast cancer lines (123).



regulators upstream of Erk activation cascade. These include gain of function mutations in RAS (typically K-RAS) that stimulate cell proliferation in 30% of all cancer and 90% of pancreatic adenocarcinoma patients. Generally, mutations in the P loop (G12) or catalytic region (Q61) of K-RAS renders K-RAS resistant to GAP inactivation. Downstream from RAS, B-RAF is commonly mutated in melanoma (39%) and papillary thyroid (38%) carcinomas. This results in hyperactivation of the closely related C-RAF as described above. However, RAS and RAF-activating mutations are seldom seen in HNSCC, suggesting Erk activation is more likely due to integrin engagement, EGFR activation or other factors (220).

Given the central importance of Erk activity in human cancers, several pharmacological agents targeting the Erk 1/2 signaling pathway have been generated to date. Selumetinib (AZD6244, utilized in Study 3) inhibits Erk 1/2 via inhibition of MEK by functioning as a non-competitive ATP-binding inhibitor. While well tolerated in early phase I clinical trials, no significant benefit in progression free survival was observed in colorectal or NSCLC patients treated with selumetinib in phase II trials (220-223). Other MEK inhibitors in clinical or preclinical trials are CI 1040 (PD184-352) and U0126, which demonstrate some efficacy in preclinical work, but to date but have not been extensively tested in the clinic (220). Sorafenib, an inhibitor designed to target all major RAF isoforms, has shown some efficacy in a phase II trial of hepatocellular carcinoma, but did not increase the overall survival of patients in a phase III trial (220,224,225). The overall ineffectiveness of MEK and RAF inhibitors in the clinic suggests that alternative MAPK pathways are likely compensating to overcome Erk 1/2 inhibition, or Erk is being activated by alternate mechanisms.

### **Src: The first oncogene.**

Over a century ago, Peyton Rous discovered the first oncogene that caused cancer in chickens (226). Termed v-Src, the human homolog c-Src was discovered years later and was subsequently identified as a proto oncogene in human cancers (227). Src is one member of a kinase family that incorporates a number of related non-receptor tyrosine kinases including the closely homologous members Yes, and Fyn in epithelia. Src modulates several cellular processes, including cytoskeletal organization, cell-cell

interactions, cell-matrix adhesion, EMT, migration and invasion (228,229). Src has a modular molecular structure, consisting of an N-terminal Src homology 4 (SH4) domain that is myristoylated to allow membrane association (230-232). SH3 and SH2 domains are adjacent to the SH4 domain and function to mediate inactivating intramolecular interactions, as well as for binding to Src adapter and substrate proteins. The SH1 domain contains the catalytic region at the C-terminus (Figure 5) (229,231,233,234).

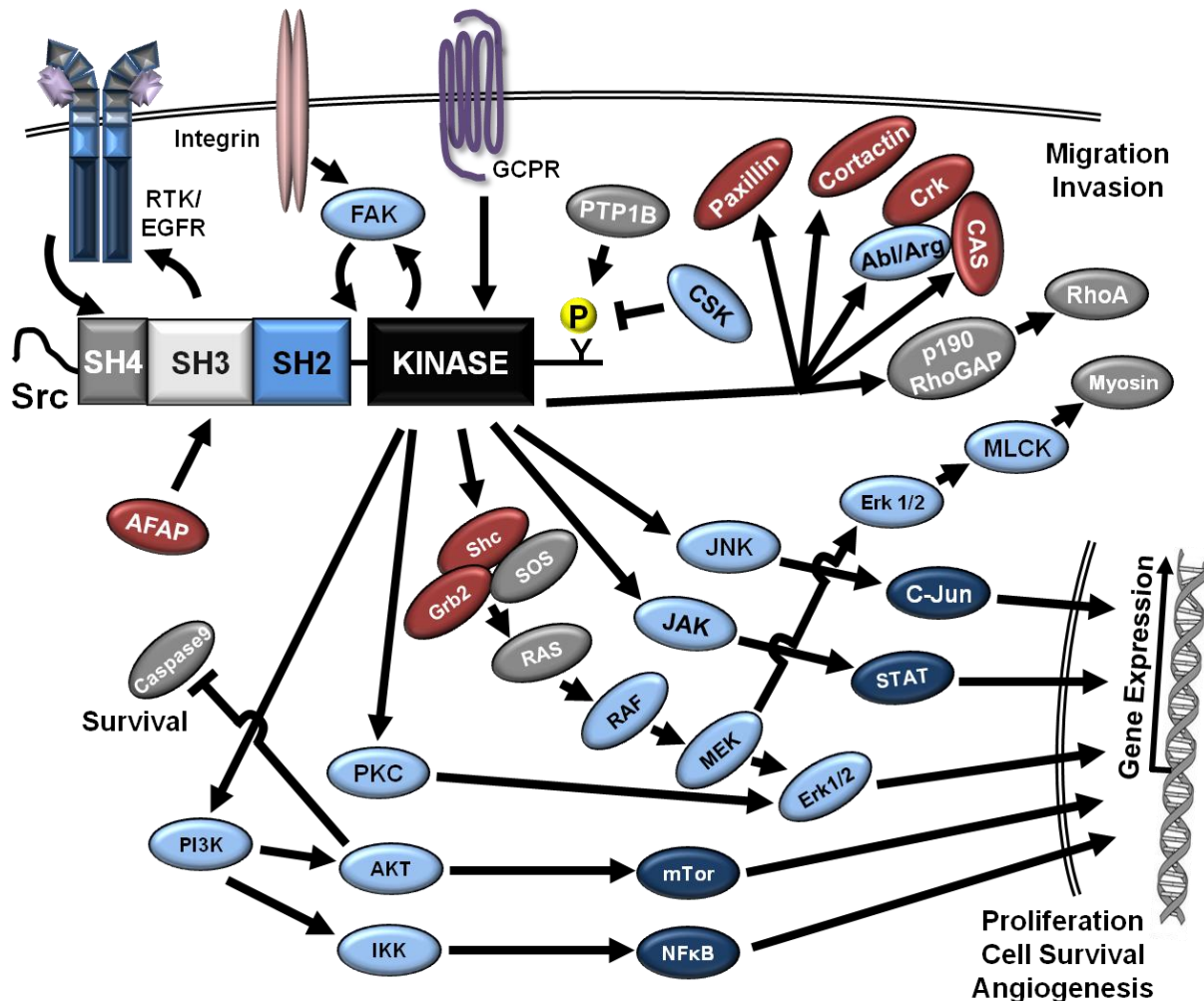
Within the C-terminal tail is a critical tyrosine residue (Y527) phosphorylated by C-terminal Src kinase (CSK). Phosphorylated (p)Y527 binds the SH2 domain to form a stable interaction that maintains Src in an inactivate state (235,236). Inactivation is also reinforced by SH2/SH1 and SH3/linker region interactions (237). Src activation is accomplished through a multi-step process initiated by the binding of substrates and/or scaffolding proteins to the SH2 or SH3 domain to disrupt intramolecular interactions between the catalytic and linker regions. Activation is further achieved through the action of the tyrosine phosphatases; PTP1B, SHP 1/2, PTP $\alpha$ , which dephosphorylates Src pY527, fully releasing the C-terminal tail from the SH2 domain to allow “opening” of Src into the active conformation (238-241). Full activation is achieved by subsequent autophosphorylation of tyrosine 419 in the kinase domain (237). In cellular contexts, integrin engagement stimulates formation of FAK/Src or p130CAS (CAS)/Src complexes through Src SH2 domain binding, achieving similar opening of the kinase and subsequent Src activation (242). Alternatively, Src activation occurs downstream of G-coupled protein receptors (GPCR) or the receptor tyrosine kinases PDGFR, EGFR, FGFR, c-Met, and HER (165,243-250). Src phosphorylates and activates receptor tyrosine kinases (e.g.; EGFR) and FAK, creating positive feedback loops that enhance Src activity.

Src mediates cellular invasion and migration by phosphorylating several key components involved in these processes. Phosphorylation of paxillin, p130CAS, and FAK disrupt focal adhesions, enhancing adhesion turnover and subsequent migration (242,251-254). Src also hinders contractility by activation of p190RhoGAP (a Src substrate), inhibiting RhoA/Rho kinase/myosin light chain II (MLC II) mediated contraction (255-257). Alternately, Src induces RAS-activation to stimulate Erk 1/2

activation and phosphorylation of myosin light chain kinase (MLCK)/MLC II, increasing contractility and disassembly of focal adhesions (258,259).

Src phosphorylates several key proteins involved in invadopodia assembly, maturation, and disassembly. Src phosphorylates and regulates AMAP1 (Arf6 effector protein) and Tks 4/5, both events that serve to modulate invadopodia actin polymerization and localization of proteins that initiate invadopodia assembly. AMAP1 binds and localizes CIN85 and Cbl to invadopodia, increasing ECM degradation. AMAP1 also binds cortactin and is required for driving breast cancer cell invasion (260-263). Following Src phosphorylation, Tks 4/5 activates NADPH oxidase to stimulate reactive oxygen species (ROS) production, creating a possible positive feedback ROS-induced activation of Src. Tsk 5 also recruits several key invadopodial proteins including cortactin and Nck responsible for regulating invadopodia function (34,39,40,264-268). In addition, Src mediates invadopodia maturation via regulation of MMP production and secretion by modulating PI3K, MAPK, and JAK/STAT activation (269-271). Src modulates vesicle trafficking through phosphorylation of endophilin A2, Cdc42-interacting protein (CIP4), and dynamin2. Src phosphorylation of CIP4 (Y471) inhibits CIP4 mediated endocytosis of MT1-MMP, which results in elevated surface expression of MT1-MMP, increasing invadopodia proteolytic activity (272). Src phosphorylation of Endophilin A2 tyrosine 315 functions in a similar manner, increasing invadopodia activity by inhibiting endophilin-induced MT-MMP endocytosis (273). In addition, dynamin requires Src phosphorylation at tyrosine sites 231/597 to mediate clathrin-coated endocytosis of MT1-MMP (274-277).

Cortactin tyrosine phosphorylation is crucial for invadopodia maturation, where Src regulates cortactin phosphorylation directly or indirectly via Abl/Arg family kinases (37,41,110,111,115-119). How Src controls invadopodia disassembly is not as clear, but Src phosphorylation of paxillin Y31 and Y118 promotes invadopodia disassembly, providing at least one mechanism (278,279). Calpain 2 and PTP1B function together to regulate invadopodia function, where calpain induces invadopodia turnover potentially via Pyk2, WASP, and talin cleavage (Figure 5) (280,281).



**Figure 5. Schematic diagram of Src, activation and downstream signaling pathways.**

Overview of Src-based signaling in invasion and proliferation. Src becomes activated as described in the main text, where it stimulates several signaling cascades including RAS/RAF/MEK/MAPK, JAK/STAT, and PI3K/Akt cascades regulating transcription, proliferation, cell survival and angiogenesis. Src also phosphorylates several key components involved in contractile and mesenchymal-based migration and invasion.

Src overexpression frequently occurs in HNSCC, where its activation correlates with poor prognosis (282). Src activation can be enhanced via several mechanisms that occur in HNSCC and other cancer types, including downregulation of CSK expression, PTP1B overexpression, activation of receptor tyrosine kinases, or activation of integrins

through ECM alteration (283). Importantly, ionizing radiation, which is frequently utilized as first line therapy in HNSCC and other cancers, stimulates Src activity due to the generation of reactive oxygen species (ROS) increasing the potential for triggering invasion and metastasis in radiation-resistant and recurrent tumors (284).

The inhibition of Src activity has become an important pursuit for molecular therapeutics since Src is a key regulator of many of processes utilized in tumorigenesis. Dasatinib, saracatinib, and bosutinib are three Src inhibitors undergoing phase I and II trials as single agents or combination therapy with conventional chemotherapeutics. These inhibitors all function in a similar manner by blocking ATP binding to the SH1 catalytic domain and have off-target effects, one of which includes serendipitous inhibition of Abl family kinases (283,285). Dasatinib (BM354825) treated HNSCC cell lines display reduced activation of Akt and Erk 1/2, resulting in impaired Bcl-2 expression, growth inhibition and increased apoptosis (286,287). In recent studies, dasatinib treatment inhibited invasion and metastasis of pancreatic cancer cell lines (288,289). Preclinical trials with dasatinib-treated breast cancer cell lines showed reduced proliferation, migration and invasion (290-292). However, recent phase II trials with breast cancer demonstrated minimal added benefit with dasatinib treatment (293-295). Our laboratory (Study 2) and others have demonstrated that saracatinib (AZD0530) treatment inhibits Src and FAK activity in HNSCC cell lines, causing reduction in proliferation and migration *in vitro* and *in vivo* (296,297). In several studies, saracatinib treatment inhibited pancreatic tumor xenograph growth and downstream phosphorylation of the Src substrates FAK, paxillin, and STATs (298,299). Use of saracatinib in a phase II trial also demonstrated minimal efficacy in patients with advanced melanoma (300).

#### **Abl: A kinase with dichotomous roles.**

Abl (Abl1, c-Abl) and Arg (Abl2) are non-receptor tyrosine kinases with 90% homology in the N-terminal region but with extremely variable C-termini (< 28% homology) (301). The N-terminal domain, similar to Src, contains a capped region that may be post-translationally modified by myristoylation, generating two isoforms (Abl1a and Abl1b) (302,303). Adjacent to the capped region are highly conserved SH3 and SH2 domains (304). Attached by a linker region to the SH2 domain, a tyrosine kinase domain targets



substrates with (L/I/V)-Y-x-x-P consensus sequence (305). Abl family kinases have over one hundred identified and potential substrates (306). Unlike other Src family kinases, Abl family kinases have a C-terminal region that consists of several proline-rich regions (Abl has four, Arg three) interspersed with three nuclear localization sequences (Abl only) (307,308). Abl and Arg diverge structurally in the C-terminal region, where Abl consists of a DNA binding region, a G-actin binding domain, and a F-actin binding region with an embedded nuclear export sequence (309-312). The Arg C-terminus includes two F-actin binding domains, a G-actin binding domain and a microtubule binding domain (Figure 6) (313,314). These differences confer some of the divergent functions for Abl and Arg in the cell. Arg is solely a cytoplasmic protein that functions to regulate actin-based lamellipodia and invadopodia, but additionally bundles actin filaments and connects actin networks with microtubules. Abl shuttles between the cytoplasm, where it also regulates dynamic actin structures, and nucleus where it is key in governing transcription, cell cycle progression and DNA damage response (315,316).

Abl family kinases, like Src, are tightly controlled by intramolecular interactions. Abl is held in an inactive conformation by binding of the myristoylated tail within a hydrophobic pocket in the kinase domain (302,303,317). Interactions between the SH3 and SH2 domains with the kinase domain also help hold Abl in an inactive state (304,317). Abl becomes weakly activated by disruption of intramolecular interactions following binding of substrates/ adapter proteins to the SH3 and SH2 domains (302). To achieve full kinase activity, Src phosphorylation of Abl or Abl trans-phosphorylation of Y245 in the linker region and Y412 in the kinase domain activation loop is mandatory (165,315,318-320). Additional Abl sites are phosphorylated that regulate protein stability (Y89 and Y261), kinase inhibition (Y272), activation (Y276), generation of potential SH2-binding sites (Y158, Y331, Y134, Y147, Y251, Y276), or are acetylated to control nuclear export (K730) (306).

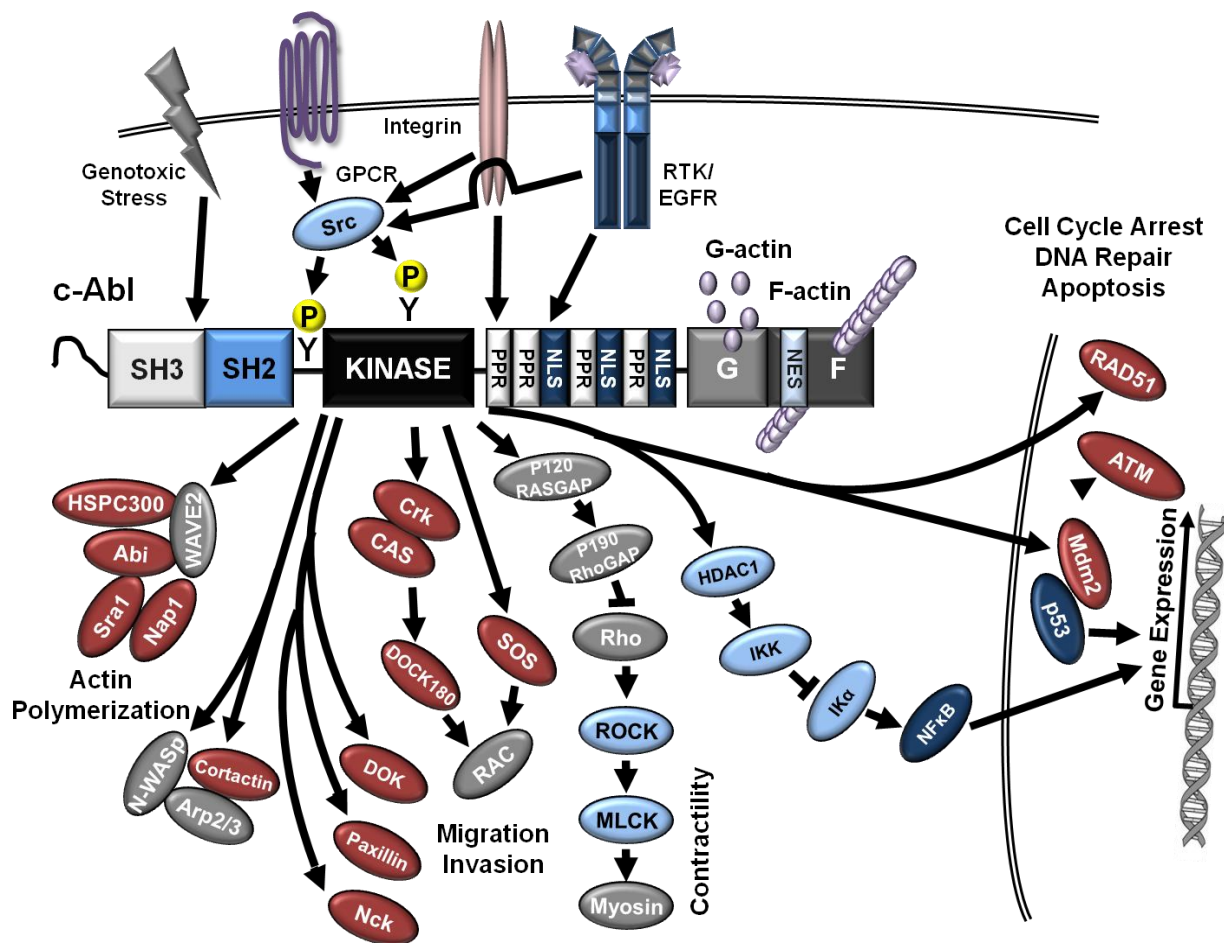
Abl family kinases are activated by several extracellular signals, EGFR, PDGFR, insulin-like growth factor 1 receptor (IGFR), integrin signaling, bacterial invasion, genotoxic and oxidative stress (165,308,321-323). Upon activation via RTK or integrins, activated Abl family kinases localize to the cell periphery, where they regulate actin-

based processes including lamellipodia protrusion and invadopodia maturation (36,37,110,111,308,315,324,325). Abl enhances actin polymerization via phosphorylation of the NPFs WAVE2, N-WASp and cortactin. Abl also phosphorylates the adaptor protein Nck1 and the actin filament capping protein Ena, enhancing Ena-mediated actin filament elongation (37,111,307,315,326,327).

Abl kinases directly and indirectly modulate cell migration and invasion. Arg phosphorylates p190RhoGAP, preventing binding to p120RasGAP and subsequent relocation of p190RhoGAP/p120RasGAP complex to cell periphery, thus preventing Rho GTPase activation reducing myosin-induced contractility (328,328,329). PDGFR mediated Rac activation is modulated by Abl; although the mechanism has yet to be defined it is likely via Abl phosphorylation of SOS1 an RASGEF (330,331). Abl-mediated phosphorylation of CrkII (Y221) and paxillin disrupts the Crk/CAS/Paxillin/FAK complex, resulting in disassembly of focal adhesions (308,332-337). Abl indirectly activates STAT3 to induce MMP1 transcription; Arg activates MT-MMP, MMP1, and MMP3 transcription in an undefined STAT3-independent manner. Additionally, Abl/Arg potentially mediate MT1-MMP endocytosis by directly binding and phosphorylating MT1-MMP (324,325). Collectively these cellular processes confer pro-invasive and anti-invasive phenotypes dependent on the substrate and cellular localization of Abl or Arg.

Nuclear localization of Abl occurs in response to genotoxic or oxidative stress (321,322,338,339). Ataxia telangiectasia mutated protein (ATM) activates Abl in response to DNA damage (340,341). Activated Abl phosphorylates murine double minute 2 (MDM2) at tyrosine 394 an E3 ubiquitin ligase that targets p53 for degradation causing the disruption of the MDM2/p53 interaction protecting p53 from degradation (342-345). Abl stabilizes ATM/p53 complex facilitating ATM serine phosphorylation of p53 and subsequent accumulation of p53 in the nucleus regulating cell cycle arrest and/or apoptosis by transcription of p21 and CDK2 or BAX, BCL2, PUMA, and NOXA (346,347). Abl initiates DNA repair mechanisms or induces apoptosis by phosphorylating and activating several key proteins including ATM, several RAD proteins, damaged DNA-binding (DDB 1/2), and DNA-activated protein kinase (DNAPK)

(348-351). Abl also regulates cell cycle progression, where phosphorylation of RNA polymerase II induces transcription of S phase genes and cell cycle progression from S to G2-M (342,352). Collectively, Abl is considered a pro-apoptotic regulator following nuclear localization and activation (Figure 6) (316,353).



**Figure 6. Schematic diagram of Abl structure, activation and downstream signaling pathways.**

Abl a non-receptor tyrosine kinase becomes activated via external stimuli; phosphorylation and binding of SH3/SH2 binding partners containing proline-rich regions or phosphorylated tyrosines. Activated Abl, in the cytoplasm, mediates processes utilized for motility and invasion (detailed in text). In the nucleus, Abl regulates transcription directly or indirectly via transcription factors controlling cell cycle arrest, DNA repair, and apoptosis.

Abl activation is attenuated by several mechanisms including de-phosphorylation by tyrosine phosphatases including PTPN1 or PTP-PEST (354). Phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) binds Abl preventing substrate binding; the PIP<sub>2</sub> inhibitory interaction is relieved by phospholipase C (PLC) hydrolysis of PIP<sub>2</sub> (164,355). Caspase-cleavage of Abl at Arginine 565 or ubiquitination and proteosomal degradation downregulate Abl signaling (338,356-358).

Abl has long been considered an oncogene in the context of the Philadelphia chromosome BCR-Abl gene product. BCR-Abl an oncogenic fusion of the Abl kinase domain with the breakpoint cluster region (BCR), giving rise to a constitutively active Abl kinase by the removal of first exon and subsequent loss of inhibitory intramolecular interactions within Abl. BCR-Abl is associated with the onset and progression of chronic myelogenous leukemia (CML) (359,360). The role of Abl in solid tumors is not as well defined, as recent work described below indicates that Abl family kinases function in a context dependent manner, possessing pro- or anti-oncogenic functions in cancer.

Research with a panel of breast cancer cell lines suggested that kinase activation and not expression of Abl family kinases correlates with breast cancer invasiveness (361,362). Mader et.al. and others have shown the loss of invadopodia proteolytic activity with imatinib-treated breast cancer and melanoma cell lines, leads to the proposal of an EGFR/Src/Arg/cortactin signaling pathway responsible for mediating invadopodia regulation (36,324). The imatinib-mediated inhibition of ECM degradation in breast cancer lines can be attributed in part to regulation of MMP 1, 3, and MT1-MMP transcription and secretion regulated by Abl and Arg (324,325). In NSCLC, inhibition of Abl with FUS1 or pharmacological agents reduced anchorage-independent growth, while Abl phosphorylation of Crk correlates with tumor cell aggressiveness (363). Collectively these studies suggest a pro-oncogenic role for Abl family kinases.

Alternately, reduction of Abl expression in HNSCC is associated with a poor patient survival (364). Inhibition of Abl expression or activity reduces invadopodia proteolytic function and invasion (115). Similar results were observed in work on breast cancer lines by other groups, where Abl mediates EphrinB1/EphR4 inhibition of cell migration and suppresses TGFβ-induced epithelial-to-mesenchymal transition (EMT) (365).

Additionally, Frasca et al. demonstrated that HGF-simulated thyroid cancer cell lines treated with imatinib had elevated Erk and Akt activity, increased motility and an EMT-like phenotype following long-term drug exposure (366). These results indicate an anti-oncogenic role for Abl.

Imatinib mesylate (Gleevec, STI571) is a competitive ATP inhibitor and first line therapy for CML patients. This compound is extremely effective, demonstrating a 90% overall response rate in patients following initial treatment (367). Second line therapies that target Abl kinase in BCR-Abl containing malignancies include nilotinib, dasatinib, bafetinib (INNO-406) and decipere (DCC-2036). These compounds have been developed to treat imatinib-refractory patients and are currently in pre-clinical, phase I and II trials (368). Nilotinib and dasatinib have a 50% complete response rate in phase II trials with imatinib-resistant CML patients (368). The efficacy of Abl inhibitors in CML and gastrointestinal stromal tumor (GIST) patients supports a pro-oncogenic role, advocating the use of imatinib for patients with solid tumors (369). Even though pre-clinical studies have demonstrated efficacy with imatinib in NSCLC and breast cancer cell lines, clinical phase I and II trials have shown no therapeutic benefits. In fact, disease progression was observed in a phase II prostate cancer trial. Furthermore, a combined HNSCC/NSCLC phase II trial was terminated early due to detrimental effects in patients treated in the imatinib-containing arm (370-374,374-376). The pro- and anti-oncogenic impact of Abl inhibition in solid tumors necessitates further investigation to determine the underlying molecular determinates responsible for the opposing oncogenic roles of Abl kinases in human cancers.

As detailed in this review, EGFR, Erk 1/2, Src, and Abl have complex signaling cascades dependent upon extracellular cues, upstream activators, cellular location, and downstream effectors. In pre-clinical studies, EGFR, Erk 1/2, Src, and Abl have been shown to be crucial for tumorigenesis in several cancer types, including breast and HNSCC. Unfortunately, the various pharmacological agents targeting these kinases (EGFR, Src, Abl) or up-stream activators (RAS, RAF for Erk1/2) typically demonstrate negligible therapeutic benefit; in fact detrimental effects have been reported in pancreatic, NSCLC and HNSCC cancer patients. Clarification and increased

understanding of the spatial and temporal molecular signaling of these key proteins is essential for improved therapy utilizing these pharmacological agents. The specific goal of this dissertation is to clarify the regulation of key signaling molecules that modulate HNSCC invasion and metastasis.

In Study 1, we elucidated the regulatory hierarchy of several proteins critical for invasion of HNSCC and breast cancer cell lines. In particular, we focused on Abl, a protein that has opposing roles in tumorigenesis. Our results demonstrate a divergent role for Abl function in invasion, where Abl kinase activity is pro-invasive in breast cancer and anti-invasive in HNSCC. Regardless of tumor type, the EGFR/Src pathway is crucial to mediate invasive regardless of divergent cellular response evoked with imatinib-treated HNSCC and breast cancer cell lines.

In Study 2, we further established the crucial role Src in HNSCC metastasis *in vitro* and *in vivo*. In addition, we examined effects of pharmacological inhibition of Src/Abl activity with saracatinib on downstream Src effectors and cellular processes important for tumorigenesis. We determined that Src/Abl inhibition reduced HNSCC proliferation and invasion in accordance with impaired invadopodia formation and MMP secretion.

Finally, in Study 3 we determined a functional role for Erk 1/2 activation and phosphorylation of the downstream substrate cortactin in HNSCC patient samples and cell lines, where we showed that Erk 1/2-mediated cortactin phosphorylation modulates HNSCC adhesion and promotes migration by prolonging lamellipodia persistence.

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# **Study 1: Ablason Kinases Negatively Regulate Invadopodia Function and Invasion in Head and Neck Squamous Cell Carcinoma by Inhibiting an HB-EGF Autocrine Loop**

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## **Abstract**

Head and neck squamous cell carcinoma (HNSCC) has a proclivity for locoregional invasion. HNSCC mediates invasion in part through invadopodia-based proteolysis of the extracellular matrix (ECM). Activation of Src, Erk1/2, Abl and Arg downstream of epidermal growth factor receptor (EGFR) modulates invadopodia activity through phosphorylation of the actin regulatory protein cortactin. In MDA-MB-231 breast cancer cells, Abl and Arg function downstream of Src to phosphorylate cortactin, promoting invadopodia ECM degradation activity and thus assigning a pro-invasive role for Abl and Arg kinases. We report that Abl kinases have an opposite, negative regulatory role in HNSCC where they suppress invadopodia and tumor invasion. Impairment of Abl expression or Abl kinase activity with imatinib mesylate enhanced HNSCC matrix degradation and 3D collagen invasion, functions that were impaired in MDA-MB-231. HNSCC lines with elevated EGFR and Src activation did not contain increased Abl or Arg kinase activity, suggesting Src could bypass Abl/Arg to phosphorylate cortactin and promote invadopodia ECM degradation. Src transformed Abl<sup>-/-</sup>/Arg<sup>-/-</sup> fibroblasts produced ECM degrading invadopodia containing pY421 cortactin, indicating that Abl/Arg are dispensable for invadopodia function in this system. Imatinib treated HNSCC cells had increased EGFR, Erk1/2 and Src activation, enhancing cortactin pY421 and pS405/418 required for invadopodia function. Imatinib stimulated shedding of the EGFR ligand heparin-binding EGF-like growth factor (HB-EGF) from HNSCC cells, where soluble HB-EGF enhanced invadopodia ECM degradation in HNSCC but not in MDA-MB-231. HNSCC cells treated with inhibitors of the EGFR invadopodia pathway indicated that EGFR and Src are required for invadopodia function. Collectively our results indicate that Abl kinases negatively regulate HNSCC invasive processes through suppression of an HB-EGF autocrine loop responsible for activating a EGFR-Src-cortactin cascade, in contrast to the invasion promoting functions of Abl kinases in breast and other cancer types. Our results provide mechanistic support for recent failed HNSCC clinical trials utilizing imatinib.

## **Introduction**

HNSCC is an aggressive disease characterized by extensive locoregional invasion and cervical lymph node metastasis (1,2). Overexpression of EGFR is common in HNSCC and correlates with enhanced invasion and nodal involvement (3-6). EGFR inhibition as adjuvant therapy in HNSCC increases survival, highlighting the importance of downstream EGFR signaling pathways in HNSCC progression (7). Downstream EGFR signaling cascades in HNSCC that promote invasion and metastasis utilize Src, Erk, PI3 kinase, Akt and STATs (8-11), supporting a role for EGFR-generated signals as important regulators of invasion promoting pathways in HNSCC.

Src kinase activation within the EGFR pathway is critical for driving tumor invasion (12,13). Elevated Src expression and activity is frequently found in HNSCC and other tumor types, where it has become a focus for targeted therapeutic design (14,15). While Src targeted drugs have been developed and demonstrate anti-invasive properties in preclinical studies (16-19), recent phase II trials demonstrate virtually no benefit for HNSCC patients with monotherapeutic Src inhibitor treatment (19,20). While combination therapy with receptor tyrosine kinase inhibitors increases efficacy (21-23), a clearer mechanistic understanding of how Src-based signaling governs HNSCC invasion is needed for the development of improved therapeutic strategies.

In carcinomas, Src activation results in the formation of invadopodia, actin-rich membraneous protrusions responsible for extracellular matrix (ECM) proteolysis, allowing tumor cells to infiltrate the stroma and vasculature (24,25). Src kinase activity regulates the cyclic development of non-degradative (pre-invadopodia) and active (mature) invadopodia (24,25). Maturation of pre-invadopodia involves recruitment and activation of matrix metalloproteinase MMP-14 to initiate ECM degradation (26-28).

Invadopodia maturation also involves increased F-actin polymerization responsible for driving plasma membrane protrusion (24,25,29). A prominent component of the invadopodia F-actin core is cortactin, an F-actin binding protein that activates the actin-related protein (Arp)2/3 complex to stimulate branched actin polymerization (30-33). Cortactin binds neuronal Wiskott-Aldrich Syndrome protein (N-WASp), a second

activator of Arp2/3 complex following phosphorylation of cortactin S405 and S418 by Erk1/2 (33-35). Cortactin modulates shifting of pre- to mature invadopodia through phosphorylation of two Src-targeted tyrosine residues (Y421 and Y470 in humans) (27,28,36). Y421 and Y470 phosphorylation results in SH2-dependent recruitment of the adaptor protein NCK1, which in turn binds and activates N-WASp to promote additional Arp2/3 activation (28,37-39). Along with tyrosine phosphorylation, cortactin domains that bind Arp2/3 and N-WASp are also required for invadopodia formation, collectively highlighting the importance of cortactin in invadopodia biogenesis and regulation (28,40,41).

The Abelson kinases Abl and Arg regulate actin cytoskeletal remodeling during motility and invasion (42-44). While Abl regulation of leukemic tumorigenesis is well established (45,46), Abl activity in solid tumors promotes multiple aspects of neoplastic progression, including increased invasion and metastasis (47-50). Activation of Abl and Arg downstream of EGFR and Src leads to direct cortactin phosphorylation at Y421 and Y470 responsible for invadopodia maturation in breast cancer and melanoma cell lines (51-55). In particular, Src-mediated activation of Arg and subsequent cortactin tyrosine phosphorylation has led to the proposal that Arg is the terminal kinase responsible for cortactin tyrosine phosphorylation required for invadopodia maturation (51,56).

Since invasive HNSCC typically contains elevated EGFR and Src activity, we postulated that downstream activation of Abl kinases may regulate invadopodia through cortactin phosphorylation in this tumor type. Paradoxically, we show that elimination of Abl expression results in enhanced invadopodia-based gelatinase activity in multiple HNSCC cells lines but not in MDA-MB-231 cells. Inhibition of Abl family kinase activity with imatinib mesylate (STI571; Gleevec) in HNSCC cells resulted in enhanced invadopodia maturation and cell invasion, whereas these processes were impaired in MDA-MB-231. Analysis of EGFR signaling indicates that EGFR and Src are hyperactivated in HNSCC compared to MDA-MB-231 cells. Introduction of active Src into Abl<sup>-/-</sup>/Arg<sup>-/-</sup> cells induced invadopodia formation, ECM matrix degradation and cortactin tyrosine phosphorylation, suggesting that elevated Src activity can bypass the requirement for Abl or Arg in invadopodia maturation. Imatinib treatment of HNSCC

cells resulted in dose-dependent activation of EGFR, Src and Erk1/2, resulting in elevated cortactin tyrosine and serine phosphorylation absent in treated MDA-MB-231 cells. Imatinib enhanced production and shedding of the EGFR ligand HB-EGF in HNSCC cells, where soluble HB-EGF stimulated HNSCC ECM degradation. Inhibition of Src and Abl kinases with the dual specificity drug saracatinib suppressed EGFR activation and ECM degradation in HNSCC, suggesting that Src is responsible for mediating the pro-invasive signals resultant from imatinib-mediated Abl family kinase inactivation. Our results indicate that in HNSCC Abl kinases serve to suppress invadopodia formation and tumor cell invasion by downregulating autocrine HB-EGF activation of the EGFR-Src-cortactin signaling pathway, in contrast to the pro-invasive function of Abl and Arg in breast and other solid tumors. These results suggest that Abl kinase function in cancer invasion is context dependent, providing molecular insight into the mechanism behind the recent failure of clinical trials with imatinib in HNSCC patients (57).

## **Results**

### **Abl expression suppresses invadopodia activity in HNSCC cells**

Since HNSCC cells form invadopodia (36,58) and Abl kinases mediate invadopodia function in other tumor types (51,53,55), we evaluated the role of Abl in HNSCC invadopodia formation and function. HA-tagged Abl localized within UMSCC1 invadopodia (Figure 1a) and in Src-expressing 1483 cells (Supplementary Figure 1a), implying a functional role. This was investigated by knockdown of Abl expression using RNA interference (RNAi). Stable UMSCC1 clones expressing an Abl-specific short hairpin RNA (shRNA2 and 3) reduced Abl expression by 65% compared to controls (Ctl2 and 8) (Figure 1b). Expression of wild-type (WT) murine Abl in shRNA cells restored expression to endogenous levels (WT5 and 6). Abl knockdown resulted in a 44% increase in gelatin degradation compared to control (Ctl) and WT lines (Figure 1c-d) without affecting the number of cells degrading ECM or the number of invadopodia per cell (Supplementary Figure 1b-c). Abl knockdown in MDA-MB-231 cells did not impact matrix proteolysis (Supplementary Figure 2a-c), in agreement with previous results (51). These data suggest that Abl expression negatively regulates invadopodia function in HNSCC cells, as opposed its invadopodia promoting role in other tumor cell types (51,53,55).

### **Imatinib treatment enhances HNSCC invadopodia activity**

To determine if Abl kinase activity was responsible for the negative regulatory effects on invadopodia function in HNSCC cells, OSC19 and UMSCC1 cells were treated with the Abl family kinase inhibitor imatinib mesylate. Analysis of Crk phosphorylation confirmed partial inhibition of Abl kinase activity in imatinib-treated lines (Figure 2a). Imatinib treatment resulted in a dose-dependent increase in OSC19 and UMSCC1 ECM degradation, demonstrating a net 2.5-3.0-fold enhancement observed at the highest tolerated concentration (15  $\mu$ M) (Figure 2b-c, 2e). In contrast, MDA-MB-231 cells treated with imatinib resulted in a 70% reduction in matrix proteolysis (Figure 2d-e). The effects on treated HNSCC and MDA-MB-231 cells is likely Abl family kinase specific, since the alternative imatinib target PDGFR is not expressed in these lines



(Supplementary Figure 3). These data indicate that imatinib impairment of Abl family kinase activity in HNSCC relieves the inhibitory effect of Abl on invadopodia function, promoting ECM degradation.

### **Imatinib treatment stimulates HNSCC invasion**

To determine if imatinib-enhanced HNSCC invadopodia activity corresponds with increased invasive behavior, 3D invasion assays were conducted using tumor spheroids embedded in collagen I. OSC19 and UMSCC1 cells treated with 10  $\mu$ M imatinib resulted in enhanced invasion, whereas invasion of imatinib-treated MDA-MB-231 cells was inhibited after 24 h (Figure 3a-c). Average invaded distances were increased by 100  $\mu$ M for OSC19 and 204  $\mu$ M for UMSCC1 cells, while MDA-MB-231 invasion was suppressed by 47  $\mu$ M (Figure 3d). These results indicate that imatinib treatment has opposing effects on tumor cell invasion, enhancing HNSCC cell invasion while impairing the invasiveness of MDA-MB-231.

### **Activated Src can bypass Abl and Arg to promote invadopodia formation**

To address the contrasting invasive roles of Abl family kinases in HNSCC and MDA-MB-231 cells, protein levels and activation of known invadopodia kinases were evaluated. Western blotting demonstrated increased Abl expression in HNSCC lines compared to MDA-MB-231 cells, whereas Arg protein levels were equivalent (Figure 4a). In spite of increased Abl expression in HNSCC cells, Abl activation was not enhanced, as evidenced by similar Crk pY221 levels between HNSCC and MDA-MB-231 cells. However, OSC19 and UMSCC1 consistently displayed increased EGFR and Src activity compared to MDA-MB-231 (Figure 4a).

The increased Src activation in HNSCC cells suggested that active Src might directly regulate invadopodia activity independent of Abl and Arg. To test this, Abl<sup>-/-</sup>/Arg<sup>-/-</sup> mouse embryo fibroblasts (DKO) were used to simultaneously evaluate the role of Abl and Arg on invadopodia function. Lack of Abl and Arg expression in DKO cells was verified by immunoblotting (Supplementary Figure 4a). EGF stimulation of wild-type (WT) MEFs demonstrated a threefold increase in cortactin pY421 over non-stimulated controls, whereas stimulated DKO cells showed a 1.5 fold increase over basal levels

(Figure 4b). While these results confirm that Abl and Arg contribute to cortactin tyrosine phosphorylation, they also indicate that additional cortactin tyrosine kinases are utilized downstream of EGFR. To determine if activated Src promotes invadopodia formation independent of Abl and Arg, WT and DKO cells transfected with activated Src (527F) were assayed for cortactin tyrosine phosphorylation, invadopodia formation and ECM proteolysis. DKO cells expressing 527F Src contained abundant invadopodia with pY421 cortactin that degraded ECM, similar to 527F Src-transformed WT cells (Figure 4c). The ability of DKO cells to degrade gelatin matrix was dependent on Src kinase activity, since kinase inactive Src (K295M) failed to promote matrix proteolysis (Figure 4d). Expression of 527F Src in DKO cells resulted in an 86% increase in matrix degradation area compared to 527F Src-expressing WT cells (Figure 4d-e, Supplementary Figure 4b). The amount of gelatin degradation per cell area in DKO 527F Src cells was inhibited by 60% following re-expression of WT Abl (Figure 4d-e), in agreement with increased matrix degradation by UMSSC1 cells with Abl knockdown (Figure 1c-d). Collectively these results indicate that Abl expression suppresses invadopodia activity in Src-transformed mouse embryo fibroblasts, and that Abl and Arg are not essential for cortactin tyrosine phosphorylation or invadopodia formation downstream of active Src.

### **Imatinib treatment enhances activation of the EGFR-invadopodia signaling pathway in HNSCC**

To determine the basis for the differential regulation of invadopodia activity and invasion when Abl/Arg activity is suppressed in HNSCC and MDA-MB-231, cells treated with imatinib were evaluated for activation of EGFR and downstream invadopodia signaling components. Increased EGFR activation was observed in imatinib-treated OSC19 and UMSSC1 cells, whereas no increase was observed in MDA-MB-231 (Figure 5a, Supplementary Figure 5a). A corresponding activation pattern was found for Src and Erk. Imatinib ablated Crk pY221 phosphorylation in MDA-MB-231, indicating that Abl/Arg-based signaling was inhibited. Crk pY221 phosphorylation was partially impaired in imatinib-treated HNSCC lines (Figure 5a) and was further downregulated

when combined with the Src inhibitor SU6656 (59) (Supplementary Figure 5b), suggesting that Crk is targeted by Src and Abl kinases in HNSCC.

Since Src and Erk regulate invadopodia in part by phosphorylating cortactin (28,36,40), cortactin tyrosine and serine phosphorylation was evaluated in imatinib-treated HNSCC and MDA-MB-231 cells. The Erk-targeted cortactin residues S405 and S418 demonstrated elevated phosphorylation in imatinib-treated OSC19 and UMSCC1 cells, corresponding with increased Erk 1/2 activation, while treated MDA-MB-231 cells did not demonstrate a substantial increase in cortactin phosphorylation (Figure 5b). Imatinib stimulated cortactin Y421 phosphorylation in HNSCC cells (Figure 5c), likely due to increased Src activation (Figure 5a). These data support activation of the EGFR-Src/Erk-cortactin pathway by imatinib in HNSCC cells that can bypass Abl/Arg inhibition to promote invadopodia activity and HNSCC invasion.

#### **Imatinib stimulates HB-EGF synthesis and promotes HNSCC invadopodia activity**

Imatinib treatment of multiple HNSCC lines results in synthesis and secretion of the EGFR ligand HB-EGF, enhancing EGFR activity (60). Lysates from imatinib treated OSC19 and UMSCC1 cells contained increased HB-EGF compared to controls, whereas HB-EGF levels in MDA-MB-231 cells were unaltered (Figure 6a). Conditioned media from imatinib-treated HNSCC cells contained increased soluble HB-EGF at levels 2.5-3.0 fold higher than from MDA-MB-231 cells (Figure 6b). Addition of recombinant HB-EGF to cells at concentrations equivalent to HB-EGF levels in imatinib-treated conditioned media enhanced ECM degradation activity by 86% in OSC19 and 30% in UMSCC1 cells, but did not increase invadopodia activity in MDA-MB-231 cells (Figure 6c-d). These results suggest that increased imatinib-induced HB-EGF expression and shedding by HNSCC cells produces an autocrine loop that stimulates EGFR activation responsible for enhancing invadopodia-mediated ECM proteolysis.

#### **Invadopodia ECM degradation promoted by impaired Abl kinase activity requires activation of EGFR and Src**

To further confirm that Src regulates HNSCC invadopodia downstream of EGFR apart from Abl kinases, pharmacological agents targeting EGFR, Src, or simultaneous Abl/Src

inhibition were evaluated for their impact on gelatin degradation in HNSCC. UMSCC1 cells treated with the EGFR inhibitor gefitinib at 5  $\mu$ M reduced EGFR activation by 60% (Supplementary Figure 6a) and matrix degradation by 50% (Figure 7a). Similar results were obtained with SU6656, where 15  $\mu$ M treatment resulted in a 65% decrease of the Src-targeted Y410 in p130CAS (Supplementary Figure 6b) and a 50% reduction in matrix degradation (Figure 7b). Treatment of UMSCC1 cells with the dual Abl kinase/Src inhibitor saracatinib, (61, 62) at 1  $\mu$ M inhibited EGFR activity by 55%, Src activity by 93%, and Abl/Arg activity by 97% (Supplementary Figure 6c). ECM proteolysis was impaired by 80% (Figure 7c). Furthermore, the enhanced ECM degradation activity in UMSCC1 cells with Abl knockdown (Figure 1b-d) was abrogated with 10  $\mu$ M SU6656 (Figure 7d; Supplementary Figure 6d). These data indicate that the enhanced matrix degradation activity promoted by targeted Abl kinase inhibition in HNSCC requires intact EGFR-Src signaling. Similar results were obtained with saracatinib in OSC19 and MDA-MB-231 cells (Supplementary Figure 7a-c).

## **Discussion**

The effects of Abl-based signaling in solid tumors are currently controversial. Several studies have determined that Abl family kinase activity directly contributes to enhancing tumor proliferation, invasion and metastasis in breast and melanoma cell lines (47,48,51,53,55). Abl family kinases also positively modulate tumorigenesis in gastric tumors (50) and non-small cell lung carcinoma (49). On the other hand, recent reports indicate that suppression of Abl kinase activity by imatinib increases breast tumor growth (86), invasion (63) and inhibits epithelial-to-mesenchymal transition (64). Imatinib also enhances thyroid cancer cell invasion (65). While these tumor stimulating findings have been attributed to use of mutationally modified Abl constructs or non-physiological levels of imatinib (53), our results comparing HNSCC lines with MDA-MB-231 cells indicate that Abl kinase inhibition by RNAi or clinically relevant imatinib concentrations yields opposite outcomes on tumor cell invasive events, suggesting that Abl kinases function to suppress HNSCC invasion by reducing invadopodia matrix degradation activity driven through the EGFR-Src-cortactin pathway.

Src activation is central to invadopodia formation and maturation, where phosphorylation of numerous downstream cytoskeletal proteins is required for invadopodia biogenesis and ECM proteolytic activity (24,25). Cortactin phosphorylation downstream of Src is involved in all stages of invadopodia formation (28,36,37,41). Src, Abl and Arg directly phosphorylate cortactin (52,66,67), indicating that these kinases can promote cortactin-based Arp2/3 nucleation activity indirectly via NCK1 and N-WASp. Src can also phosphorylate and activate Abl and Arg downstream of EGFR and other growth factor receptors (54,68) and recent work has shown that removal of Abl or Arg by RNAi prevents invadopodia formation in Src transformed fibroblasts, MDA-MB-231 breast cancer and melanoma cell lines (51,53,55). While these studies indicate that Abl and Arg are the key cortactin kinases responsible for cortactin tyrosine phosphorylation in invadopodia (Figure 8a), we show that EGF stimulation of Abl/Arg-null (DKO) fibroblasts enhances cortactin tyrosine phosphorylation, indicating that Src and/or other EGFR-activated cortactin targeting kinases phosphorylate cortactin apart from Abl or Arg. Src-transformed Abl/Arg-null fibroblasts retain the ability to degrade

ECM and contain tyrosine phosphorylated cortactin within invadopodia, demonstrating that Abl family kinases are not essential in this system for invadopodia function driven by cortactin tyrosine phosphorylation. Abl re-expression in DKO cells impaired Src-generated ECM degradation, confirming an inhibitory role for Abl in invadopodia maturation similar to that observed in HNSCC cells. These results indicate that elevated levels of Src activity commonly present in HNSCC lines (36) or due to ectopic expression of active Src forms can circumvent the requirement for Abl or Arg in regulating invadopodia activity through cortactin phosphorylation (Figure 8b).

Imatinib-stimulated HNSCC invadopodia activity and invasion is likely due to increased activation of EGFR and associated downstream signaling, since imatinib treatment enhances EGFR, Src and Erk activation in HNSCC lines while having minimal impact on EGFR signaling in MDA-MB-231 cells. Imatinib treatment of HNSCC cells results in increased cortactin phosphorylation at Src-targeted Y421 and Erk-targeted S405/S418, phosphorylation events present within invadopodia required for ECM matrix degradation activity (40,69,70). As in other HNSCC lines (60), we observed that imatinib treatment increases synthesis and release of the EGFR ligand HB-EGF from OSC19 and UMSCC1 cells at concentrations 10-fold higher than MDA-MB-231 cells. Application of HB-EGF at imatinib-treated conditioned media concentrations enhanced invadopodia activity in HNSCC but not in MDA-MB-231 cells. This suggests that HNSCC cells lacking Abl or treated with imatinib generate an autocrine loop, where increased HB-EGF synthesis and shedding in turn binds and activates EGFR to stimulate Src and Erk activation, leading to elevated cortactin phosphorylation and enhanced invadopodia ECM degradation (Figure 8b). HB-EGF induction of HNSCC invadopodia activity likely occurs in part through stimulation and secretion of MMP9 (71,72), which localizes with UMSCC1 invadopodia at sites of ECM degradation (73). While our data are congruent with these findings, it is possible that other EGFR ligands may also be upregulated by Abl kinase suppression. The increased level of EGFR overexpression in HNSCC cells would render this tumor type more responsive to soluble HB-EGF than cell types that contain lower EGFR levels (e.g., MDA-MB-231), which may be the underlying reason for the differential response to Abl knockdown, imatinib and HB-EGF in our analyzed cell lines. Whether such a scenario applies to other EGFR overexpressing cancers

and/or tumor types that display pro-invasive behavior in response to Abl kinase suppression will be important to determine.

How Abl kinase inhibition promotes HB-EGF synthesis and shedding in HNSCC is unclear. In addition to cytoskeletal regulation, Abl is a nuclear kinase and work in Abl-null and imatinib-treated fibroblasts indicates that Abl functions to suppress NF- $\kappa$ B (nuclear factor- $\kappa$ B) activity through stabilization of the NF- $\kappa$ B regulator HDAC1 (74). Elevated NF- $\kappa$ B activity results in increased HB-EGF expression and EGFR activation (75), providing a link between Abl activation and negative regulation of HB-EGF expression. High HB-EGF expression is linked to poor clinical prognosis in HNSCC (76) and low Abl expression in HNSCC correlates with late stage tumors with poor outcome (77), supporting a potential connection between Abl activity and HB-EGF levels in driving HNSCC progression.

Pathway analysis with inhibitors of invadopodia signaling components confirmed that EGFR and Src activation in HNSCC is central to driving invadopodia-based ECM degradation. Simultaneous inhibition of Src and Abl kinases decreased matrix degradation by UMSCC1 and OSC19 cells, reinforcing the point that the elevated invadopodia activity in HNSCC resultant from Abl kinase inhibition requires concurrent Src activation. While preclinical studies on HNSCC lines has shown that saracatinib and the related inhibitor dasatinib impair cell invasion and display potent anti-tumor effects (9,17), phase II trials in HNSCC patients with either drug as a single agent yielded no benefit in spite of apparent Src inhibition (78,79). In contrast, a phase II trial with imatinib administered to HNSCC and NSCLC patients was closed early due to lack of efficacy and antagonistic effects, with a patient subset displaying a worse clinical outcome in response to imatinib (57). While not directly evaluated, these results support a role for Src inhibition in counteracting the pro-invasive effects of Abl kinase that would result in increased patient tumor progression. Although some response has been achieved with imatinib in solid tumors with combination approaches (78,80-82), our results further emphasize the importance of careful patient selection and exclusion criteria for using imatinib or other Abl kinase inhibitors in HNSCC and other solid tumors that display similar characteristics.

We have determined that Abl kinases have a context-dependent role in regulating invadopodia function and tumor invasion. In HNSCC cells Abl serves to suppress invadopodia ECM degradation and tumor invasion by preventing HB-EGF synthesis and extracellular shedding, where it is capable of activating the EGFR/Src/cortactin signaling pathway to accelerate invadopodia-based ECM degradation and tumor cell invasion. This is in contrast to MDA-MB-231 cells, a cell line commonly used to analyze invadopodia and invasive signaling, where Abl kinase inhibition prevents invadopodia activity and impairs invasiveness. These results stress the need for further mechanistic insight into the signaling processes that regulate the pro- and anti-oncogenic roles of Abl in solid tumors in order to prevent detrimental affects of imatinib treatment in ongoing and future patient trials.



## **Material and Methods**

### **Plasmid constructs**

The lentiviral vector pLL5.0 (83) was used for Abl knockdown by subcloning a shRNA targeting human Abl oligonucleotide, (5'GCTCCGGGTCTTAGGCTAT3'; (84)) with HpaI and XhoI sites. For Abl knockdown-rescue experiments, the resulting vector was modified to encode a 6X-HA epitope tag using BamHI and SbfI sites. Human Abl cDNA was PCR amplified from pMSCV-puro Abl (85) and subcloned into EcoRI and BamHI digested pLL5.0 6X-HA. Src-GFP constructs (WT, 527F, and 295M) were used as described (36).

### **Cell culture, lentiviral infection and transfection**

UMSCC1, OSC19 and 1483 cells were maintained as described (36).  $Abl^{-/-}/Arg^{-/-}$ , wild-type MEFs, NIH3T3 and HEK 293T/17 cells were cultured as before (86). MDA-MB-231 cells were cultured in alpha Minimum Essential Media (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan UT), and 1% penicillin-streptomycin.

UMSCC1 cells stably infected with pLL5.0 6X-HA, pLL5.0 6X-HA Abl shRNA or pLL5.0 co-expressing Abl shRNA and HA-tagged Abl were generated by puromycin selection following standard methods. 1483,  $Abl^{-/-}/Arg^{-/-}$  or wild-type MEF cells transiently expressing CMV-Src or HA-Abl constructs were transfected with the Nucleofector I device (Amaxa Biosystems, Berlin, Germany).

### **Western blotting, antibodies and immunoprecipitation**

Western blotting of cell lysates was conducted as described (73). Antibodies used were: anti-Src clone GD11 (1:1000; EMD Millipore, Billerica, MA), anti-pY418 Src (1:1000; Invitrogen, Carlsbad, CA), anti-p130CAS (1:1000; BD Biosciences, San Jose, CA), anti-pY410 p130CAS (1:1000; Cell Signaling Technology, Danvers, MA), anti-cortactin clone 4F11 (1 µg/ml, (86)), anti-pY421 cortactin (1:500; BD Biosciences), anti-pS405 cortactin (1:2000; (70)), anti-pS418 cortactin (1:500; (70)), anti-ERK1/2 clone C-14 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-pERK1/2 (1:1000; Santa

Cruz), anti- $\beta$ -actin (1:10,000; EMD), anti-HA (1:200; Covance, Berkley, CA), anti-Crk (1:1000; BD), anti-pY221 Crk (1:1000; Cell Signaling), anti-PDGFR (1:200; Cell Signaling), anti-EGFR (1:1000, BD), anti-pY1068 EGFR (1:1000; BD), anti-Arg (1:500; EMD), anti-HB-EGF (1:200; EMD), and anti-Abl clone 8E9 (1:500; BD). Blots were quantified as described (73).

Immunoprecipitation was conducted from cells lysed in RIPA buffer (69). Clarified lysates (250  $\mu$ g) were incubated with 5  $\mu$ g anti-cortactin antibody 4F11 for 2 h at 4 °C. Immune complexes were captured by incubation with 30  $\mu$ L Protein A/G beads (ThermoFisher Scientific, Rockford, IL) for 1 h, washed with RIPA and analyzed by Western blotting.

### **Invadopodia matrix degradation assays and fluorescence microscopy**

Cells were plated on Oregon Green 488-conjugated gelatin (Invitrogen, Grand Island, NY) coated coverslips (27,87). In cases of inhibitor treatment, cells were allowed to attach for 1 h, then serum starved for 12 h in the presence of 10  $\mu$ M GM6001 (Sigma) and either imatinib mesylate (LGM Pharmaceuticals, Boca Raton, FL), saracatinib (AstraZeneca, Alderley Park, Cheshire, United Kingdom), gefitinib (AstraZeneca) or SU6656 (EMD). Serum-free media was replaced with complete media containing 10% FBS and kinase inhibitors for 12 h. Cells were rinsed in PBS, fixed in fresh 4% paraformaldehyde and labeled as described (36). Primary antibodies used were anti-cortactin clone 4F11 (1:500), anti-cortactin EP1922Y (1:500; Novus Biologicals, Littleton, CO), anti-cort-pY421 (1:500), anti-Src GD11 (1:500), anti-Abl clone 8E9 (1:200), anti-GFP (1:500; Invitrogen), and anti-HA (1:200). Primary antibodies were visualized using Alexa Fluor 405 and 647 conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:2000; Invitrogen). F-actin was visualized using rhodamine-conjugated phalloidin (1:500; Invitrogen). Cells were mounted with ProLong Gold (Invitrogen) and images were acquired with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss MicroImaging, Thornwood, NY). Gelatin degradation was quantified as described previously (87). In brief,  $\geq 90$  transiently transfected and  $\geq 300$  lentiviral infected or inhibitor-treated cells evaluated for each condition. For therapeutic treatments and RNAi stable cell lines, the area of degradation and cell area was

determined by analyzing the intensity of degraded gelatin or F-actin respectively in an entire field of view utilizing ImageJ software. For transient transfections, the area of degradation and cell area was determined by Image J software on an individual cell basis. The number of invadopodia per cell ( $n \geq 50$ ) and number of cells degrading matrix ( $n \geq 100$ ) were determined on each independent experiment ( $n = \geq 3$ ) (36).

### **3-D spheroid invasion assays**

Cells were labeled with Vybrant® Dil (Invitrogen). 96 well plates were coated with 100  $\mu$ L of 1.5% noble agar (BD Biosciences, Sparks, MD) in Dulbecco's PBS.  $1 \times 10^3$  (OSC19),  $5 \times 10^3$  (UMSCC1), or  $2.5 \times 10^3$  (MDA-MB-231) labeled cells were plated into individual wells for 48 h to form spheroids. Two spheroids were transferred to a microcentrifuge tube and centrifuged at  $1000 \times g$  for 3 min. The media was aspirated and replaced with 500  $\mu$ L of 2 mg/mL rat tail collagen I (BD). The spheroid mixture was transferred to an individual well of 24-well plate pre-coated with 400  $\mu$ L solidified 2 mg/mL collagen I. Plates were incubated for 1 h at 37 °C then overlaid with 1 mL of complete media. Spheroid invasion was visualized by fluorescence microscopy (Zeiss, Axiovert 200M) to establish the central z-axis (0 h) and imaged at 0 and 24 h by phase contrast microscopy. Spheroids were pretreated for 24 h and maintained in media with DMSO vehicle or 10  $\mu$ M imatinib. Maximal radial distances for invaded cells was calculated using Axiovision 4.6 software (Zeiss).

### **HB-EGF ELISA assays**

HB-EGF specific enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's protocol (Abcam, Cambridge, MA). Cells were treated with imatinib (10 $\mu$ M) or DMSO for 12 h, washed with PBS and incubated for 24 h in serum-free media with imatinib or DMSO. Conditioned media was concentrated to 500  $\mu$ L, and 100  $\mu$ L of media incubated overnight at 4°C in HB-EGF antibody-coated microplate strips. Absorbance values were obtained at 450 nm with a Biotek Synergy H1 Hybrid Reader (Winooski, VT). Standard curves were generated and results normalized to total cellular protein concentration for comparison across different cell lines.

## Statistical analysis

Differences in mean values between groups were evaluated using Students *t*-test and significance was determined at  $P < 0.05$ . Scale bars represent confidence intervals (C.I.).

## **Conflict of Interest**

The authors declare they have no competing financial interests in relation to the work described.

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## **Figure Legends**

**Figure 1** Abl expression inhibits invadopodia activity in HNSCC cells. **(a)** UMSCC1 cells transfected with empty vector (HA-Ctl) or HA-tagged Abl (HA-Abl) were plated on Oregon Green 488-gelatin coated coverslips (pseudocolored white) and incubated for 24 h. Cells were fixed and labeled with anti-HA (blue), anti-cortactin (green) and rhodamine-phalloidin (F-Actin; red). Boxed areas and corresponding insets denote regions of HA-Abl localization with invadopodia markers and regions of matrix degradation. **(b)** Western blot analysis of Abl expression. Cell lysates (100 µg) from UMSCC1 control (Ctl), shRNA Abl knockdown (shRNA) and wild-type (WT) Abl rescued shRNA cells. Parallel blots were probed for β-actin to confirm equal protein loading across all lines. Numbers denote different independent clones. **(c)** Representative confocal images of gelatin matrix degradation assays conducted with the indicated UMSCC1 clonal cell lines described in **(b)**. Cells were plated on Oregon Green 488-gelatin coated coverslips (pseudocolored white) for 9 h, fixed and labeled with an anti-cortactin antibody (green) and rhodamine-phalloidin (F-Actin; red). The amount of gelatin degradation per cell area from four independent experiments was quantified in **(d)**. Data are represented as mean ± C.I.; \*\*  $P \leq 0.01$ . Scale bars: 10 µm in **(a)**, 30 µm in **(c)**.

**Figure 2** Targeted inhibition of Abl family kinases with imatinib has divergent effects on invadopodia activity in HNSCC and breast cancer cells. **(a)** OSC19, UMSCC1 (HNSCC) and MDA-MB-231 (breast cancer) cells were treated with the indicated concentrations of imatinib (STI571) for 24 h, lysed and 25 µg of cell lysate analyzed by Western blotting with anti-pY221 Crk to determine drug efficacy. The ratio of pY221 Crk phosphorylation to total Crk levels determined by densitometry is displayed between blots. Blots are representative from three to four independent experiments for each cell line. **(b)** OSC19, **(c)** UMSCC1, and **(d)** MDA-MB-231 cells were plated on Oregon Green 488-gelatin coated coverslips (pseudocolored white) in complete media for 1 h to allow attachment. Cells were then serum-starved and treated with GM6001 (10µM) and either imatinib (STI571) or DMSO vehicle (0 µM) concomitantly for 12 h. After GM6001 washout, cells were incubated with complete media containing 10% FBS for 12 h in the



presence or absence of imatinib. Cells were fixed and labeled with an anti-cortactin antibody (green) and rhodamine-phalloidin (F-Actin; red) and visualized using confocal microscopy. Representative images showing cortactin/F-Actin labeling and gelatin degradation for each line and indicated experimental condition are shown. Insets denote areas of invadopodia activity indicated by the presence of overlapping cortactin/F-Actin puncta that co-localize with dark regions of Oregon Green 488-gelatin clearing. Scale bars: 20  $\mu$ m. **(e)** Quantification of Oregon Green 488-gelatin degradation for OSC19, UMSCC1 and MDA-MB-231 cells treated with the indicated imatinib (STI571) concentrations. Data from 3 independent experiments for each experimental condition are represented as mean  $\pm$  C.I.; \*\*  $P \leq 0.01$ .

**Figure 3** Imatinib enhances HNSCC invasion through 3D collagen matrices. **(a)** OSC19, **(b)** UMSCC1 and **(c)** MDA-MB-231 tumor cell spheroids pre-treated with 10  $\mu$ M imatinib (STI571) or DMSO vehicle (0  $\mu$ M) for 24 h were embedded in collagen I (0 h). Spheroids were further incubated in complete media without (0  $\mu$ M) or with imatinib for an additional 24 h and invasion monitored by phase contrast microscopy. White circles indicate the maximum radial distance traveled by invaded cells. Scale bars: 200  $\mu$ m. **(d)** Quantification of average invasive distance traveled by cells in each experimental condition ( $n \geq 12$  spheroids assayed per cell line and treatment). Data are represented as mean  $\pm$  C.I.; \*\*  $P \leq 0.01$ ; \*  $P \leq 0.05$ .

**Figure 4** Elevated Src activity bypasses Abl family kinase regulation of invadopodia function. **(a)** UMSCC1, OSC19 and MDA-MB-231 cells grown in complete media were analyzed by Western blotting of total cell lysates (25-50  $\mu$ g) for relative basal activation of EGFR, Src and Abl family kinases with the indicated phosphorylation-specific antibodies. Lysates were immunoblotted in parallel for total EGFR, Src, Crk, Abl and Arg protein levels. **(b)** Abl<sup>-/-</sup>/Arg<sup>-/-</sup> double knockout (DKO) and wild-type (WT) mouse embryo fibroblasts (MEFs) were serum starved overnight, then stimulated with 100 ng/mL EGF for 15 min. Control and stimulated cells were lysed, and 50  $\mu$ g of total protein assayed by immunoblotting with phosphorylation-specific antibodies against cortactin pY421 (Cort pY421) and total cortactin. The ratio of pY421 cortactin relative to total cortactin levels for each cell type and treatment are shown. **(c)** WT and DKO cells

transiently expressing constitutive active Src (527F) were plated on Oregon Green 488-gelatin coated coverslips for 12 h, fixed, and immunolabeled with anti-cortactin-pY421 (blue), anti-cortactin (green) and rhodamine-phalloidin (F-Actin; red). Cells and matrix were imaged by confocal microscopy. Insets show co-localization of pY421 cortactin within invadopodia at areas corresponding with clearing of Oregon Green 488-gelatin (pseudocolored white). DKO cells expressing GFP-tagged wild-type (WT), 527F, kinase inactive (K295M) Src, co-expressing 527F Src and wild-type (WT) Abl or wild-type (WT) MEFs expressing 527F Src were assayed for the percentage of cells degrading the ECM **(d)** and for normalized matrix degradation per cell area **(e)**. Data are represented as mean  $\pm$  C.I. from 3 independent experiments. \*\*  $P \leq 0.01$ .

**Figure 5** Imatinib activates the invadopodia kinase pathway in HNSCC cells. **(a)** OSC19, UMSCC1 and MDA-MB-231 cells grown in complete media were treated with DMSO vehicle (0  $\mu$ M) or the indicated imatinib (STI571) concentrations for 24 h. Cells were lysed and 25-50  $\mu$ g of total cell protein were assayed by immunoblotting for activation of EGFR (pY1068), Src (pY418), Erk (pErk) and Abl/Arg kinases (pY221 Crk). Parallel blots were probed for corresponding levels of each assayed protein. Ratios of phosphorylated/total protein are shown (represented as mean from  $\geq 3$  independent experiments). All blots are representative images. **(b)** Western blot analysis of cortactin S405 and S418 phosphorylation from cells treated and prepared in **(a)** with the indicated phosphorylation-specific antibodies. A representative blot was stripped and reprobed with anti-cortactin to verify equal loading. **(c)** OSC19, UMSCC1 and MDA-MB-231 cells treated as in **(a)** were lysed and cortactin was immunoprecipitated from 250  $\mu$ g of cell extract. Immune complexes were assayed by Western blotting for cortactin Y421 phosphorylation (anti-Cort-pY421). Parallel blots with anti-pan-cortactin were conducted to verify cortactin immunoprecipitation.

**Figure 6** Imatinib induced HB-EGF stimulates HNSCC invadopodia ECM degradation. **(a)** OSC19, UMSCC1 and MDA-MB-231 cells treated with DMSO (0  $\mu$ M) or imatinib mesylate (STI571, 10  $\mu$ M) for 24 h were lysed and 100  $\mu$ g cell protein assayed by Western blot analysis for HB-EGF. Bracketed HB-EGF shows different HB-EGF post-translationally modified forms. Positive control (+ Ctl) recombinant HB-EGF ectodomain

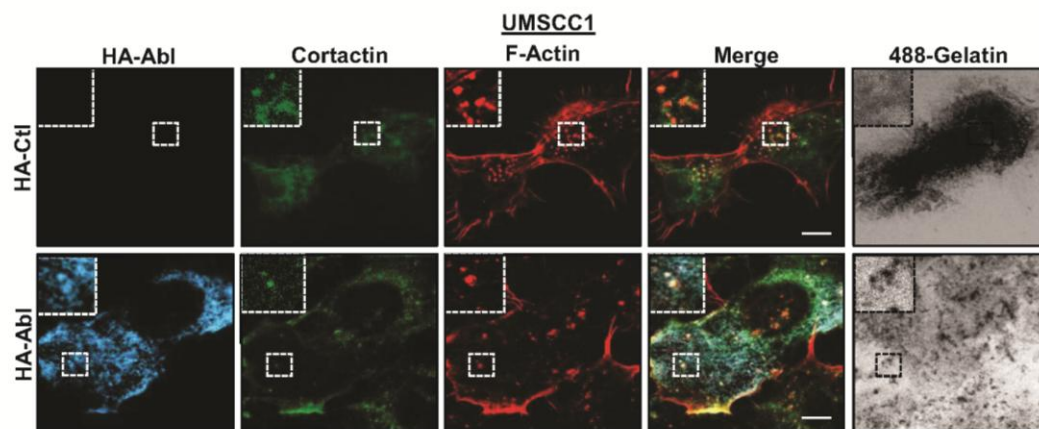
is denoted by an asterisk (\*). A parallel blot was probed with anti- $\beta$ -actin to confirm equal protein loading. **(b)** HB-EGF ELISA analysis of conditioned media from cells treated with the indicated imatinib (STI571) concentrations. Concentrations of cleaved, soluble HB-EGF were adjusted in accordance with total cellular protein levels after treatment from each cell line to allow cross-comparison. **(c)** Soluble HB-EGF promotes HNSCC invadopodia ECM degradation. OSC19, UMSCC1, and MDA-MB-231 cells were plated on Oregon Green 488-gelatin coated coverslips in complete media for 2 h to allow attachment and stimulated with recombinant HB-EGF (0, 25, or 250 pg/mL) for 7 h. HB-EGF concentrations were calculated from average levels present in conditioned media of imatinib-treated cells. Cells were fixed and labeled with an anti-cortactin antibody (green) and rhodamine-phalloidin (F-Actin; red) and visualized using confocal microscopy. Insets denote areas of invadopodia activity. Scale bars: 20  $\mu$ m. **(d)** Quantification of Oregon Green 488-gelatin degradation from OSC19, UMSCC1 and MDA-MB-231 cells treated with the indicated HB-EGF concentrations as in **(c)**. Data are represented as mean  $\pm$  C.I.; \*\*  $P \leq 0.01$ ; \*  $P \leq 0.05$ .

**Figure 7** Targeted inhibition of the EGFR-Src pathway impairs HNSCC invadopodia activity. UMSCC1 cells treated with the indicated concentrations of gefitinib **(a)**, SU6656 **(b)** and saracatinib **(c)** for 24 h were fixed and evaluated for Oregon Green 488-gelatin degradation by fluorescence microscopy with rhodamine-phalloidin and anti-cortactin antibodies. **(d)** UMSCC1 control (Ctl 8), shRNA Abl knockdown (shRNA 3) and Abl-rescued shRNA cells (WT 6) were treated with vehicle (0  $\mu$ M) or SU6656 (10  $\mu$ M) and assayed for effects on Oregon Green 488-gelatin proteolysis as above. Cells and matrix were imaged by confocal microscopy and the level of matrix degradation quantified for each treatment condition. Data are represented as mean  $\pm$  C.I.; \*\*  $P \leq 0.01$ ; \*  $P \leq 0.05$ .

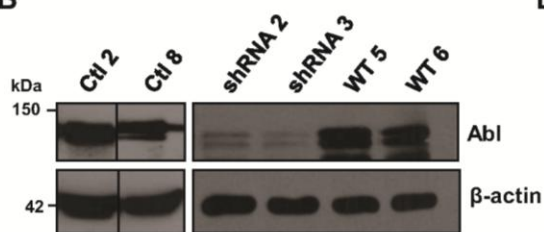
**Figure 8** Schematic diagram of EGFR-invadopodia signaling pathways altered by Abl inhibition in HNSCC. **(a)** The current invadopodia pathway depicting EGFR activation to cortactin phosphorylation as primarily determined in breast cancer cell lines. EGFR activation increases Src and Erk1/2 kinase activity, where Src stimulates Abl/Arg activation resulting in direct Abl/Arg cortactin tyrosine phosphorylation, while Erk1/2

directly phosphorylates cortactin S405/418. **(b)** In HNSCC, inhibition of Abl/Arg results in increased activation of EGFR through elevated HB-EGF synthesis and shedding, resulting in autocrine EGFR hyperstimulation. This leads to elevated Src activity that bypasses Abl/Arg and directly phosphorylates cortactin. Erk1/2 activation is also increased under these conditions, where the combined effect of enhanced Src and Erk1/2 activation increases cortactin phosphorylation and correlates with elevated HNSCC-mediated ECM degradation and invasion.

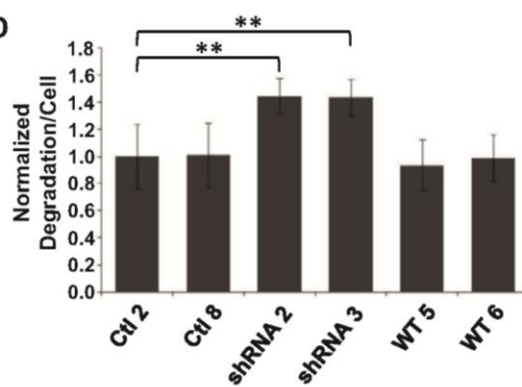
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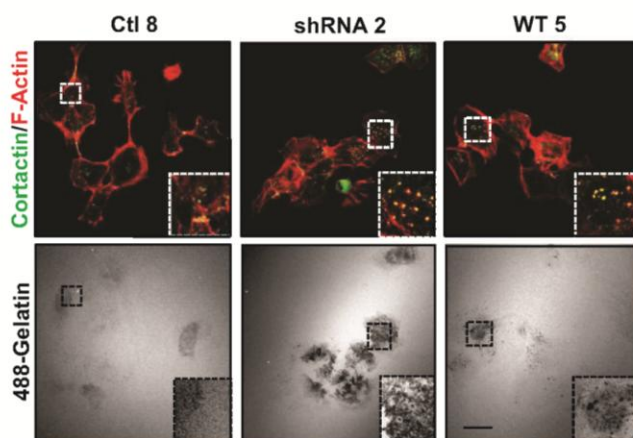
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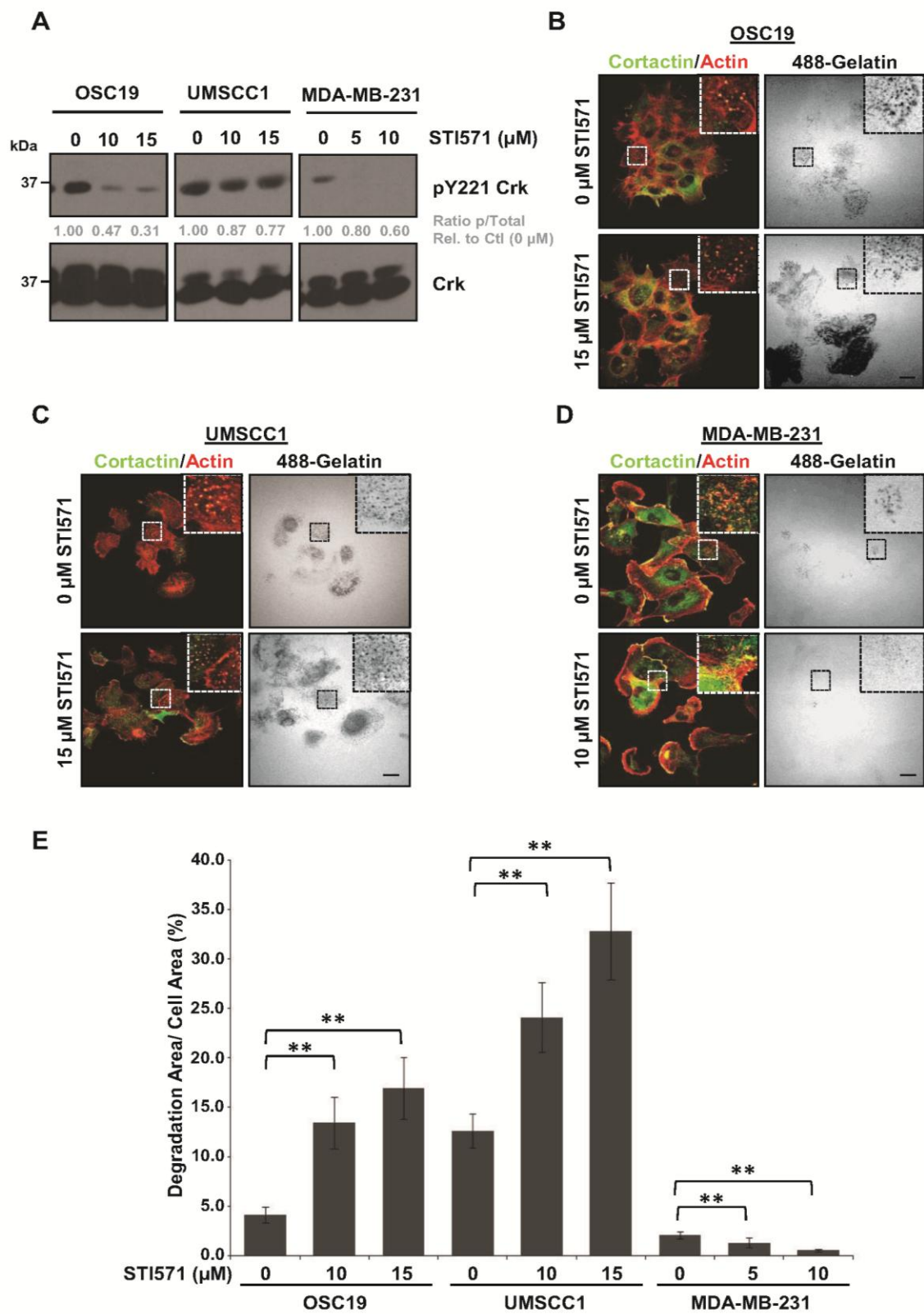
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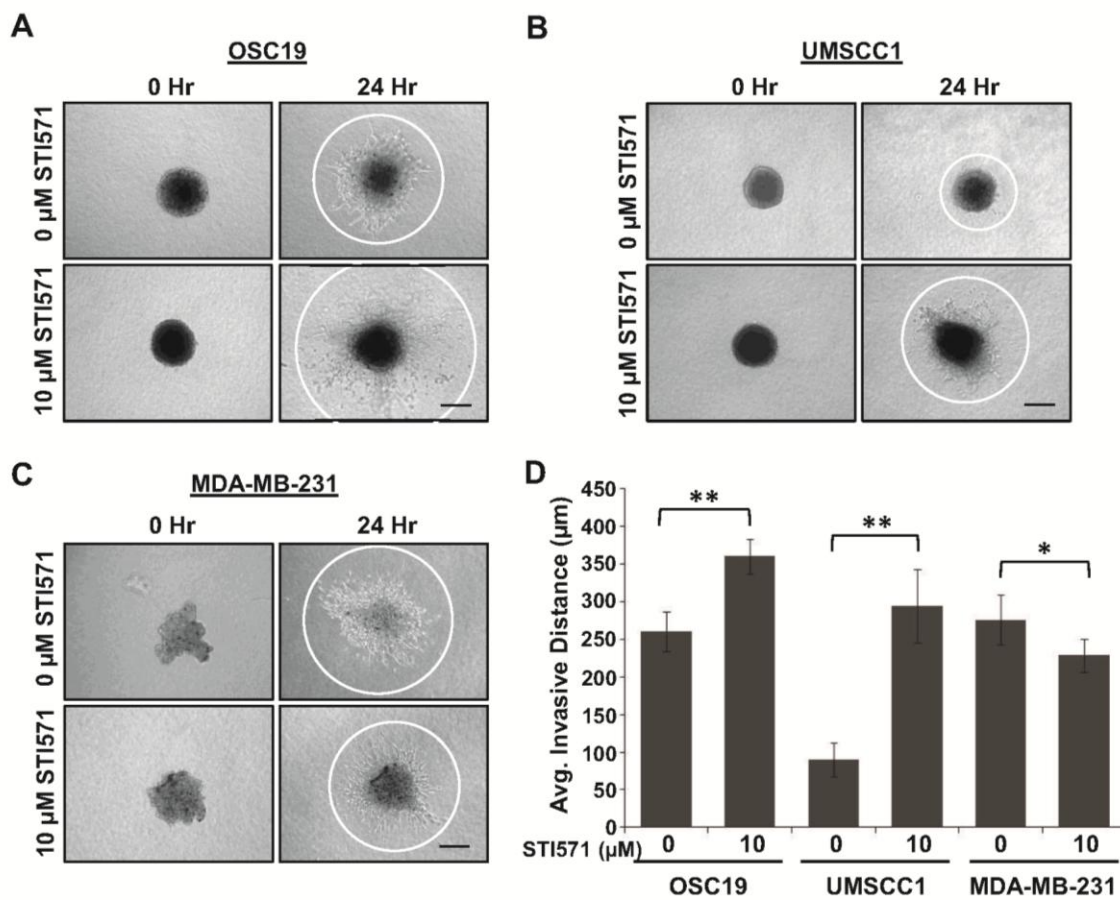
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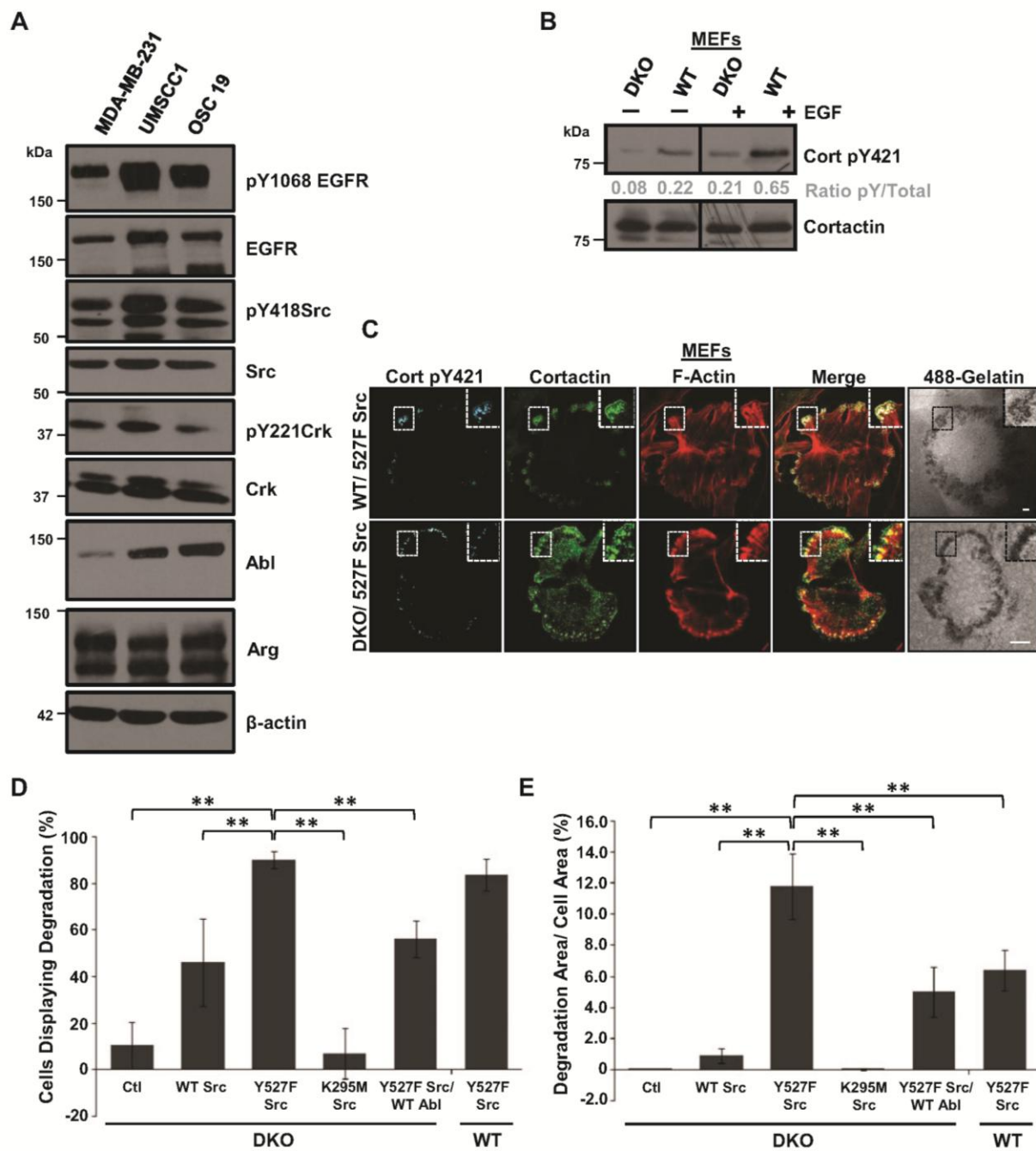
Hayes et al. Figure 1



Hayes et al. Figure 2

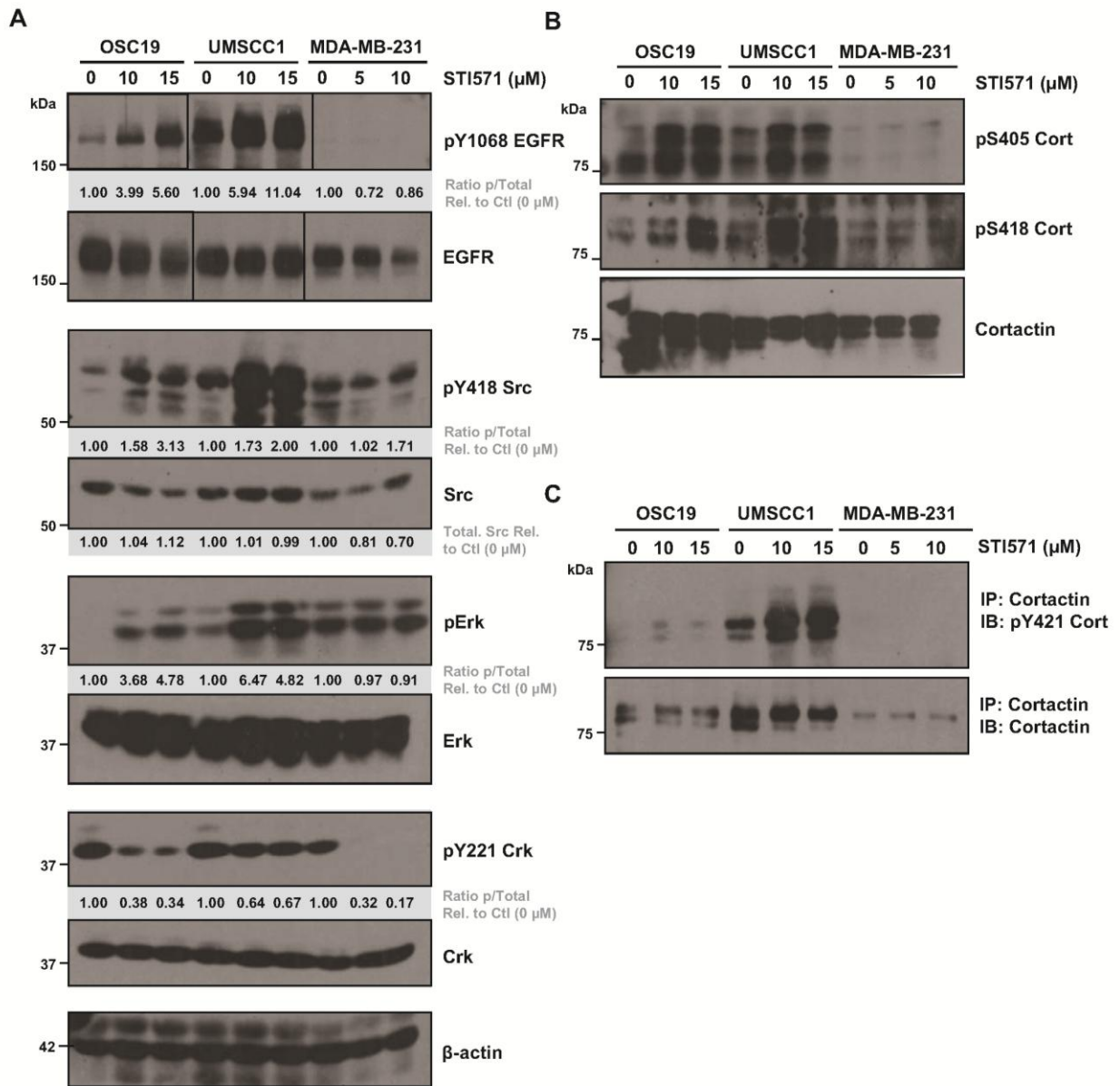


Hayes et al. Figure 3

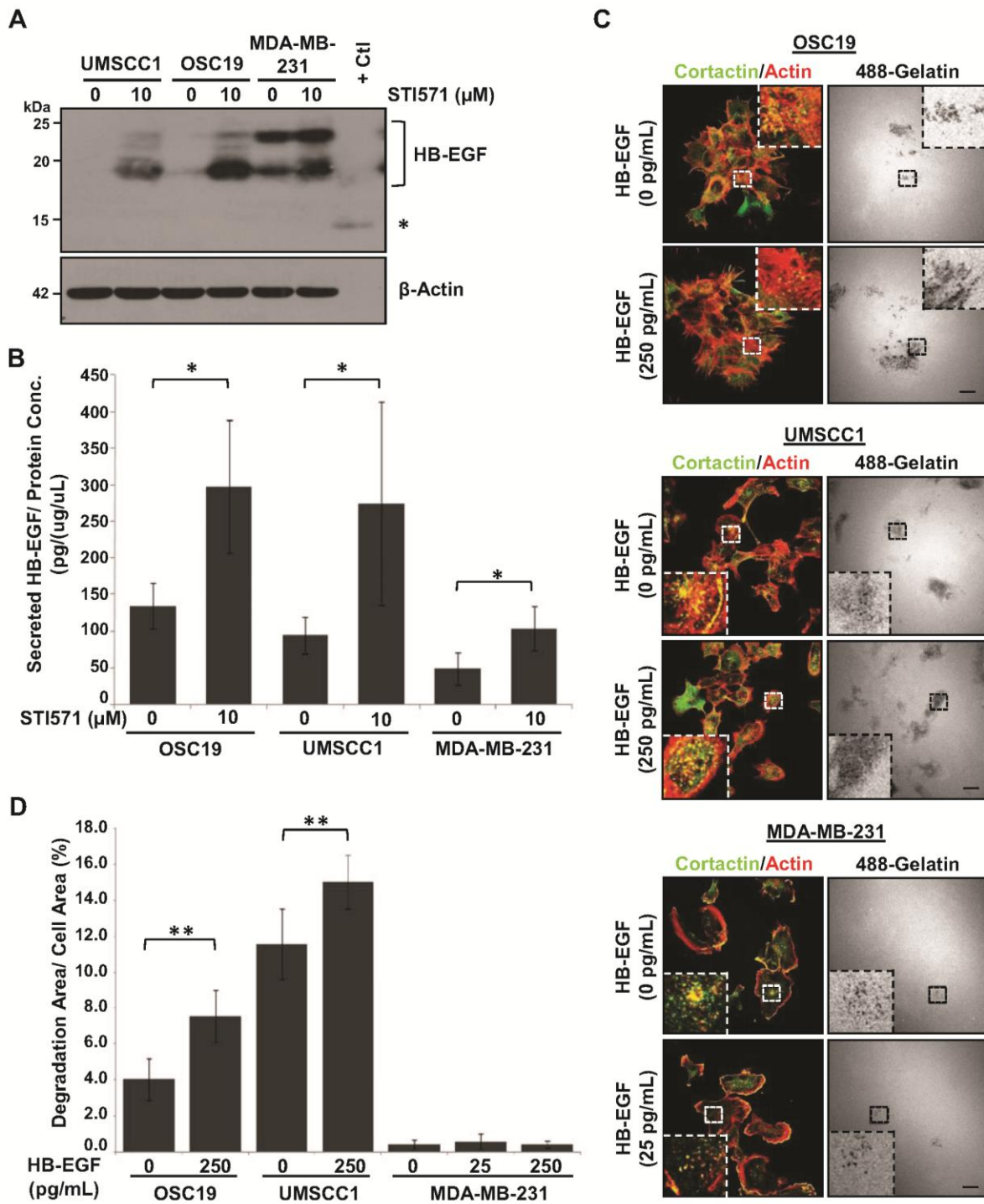


Hayes et al. Figure 4

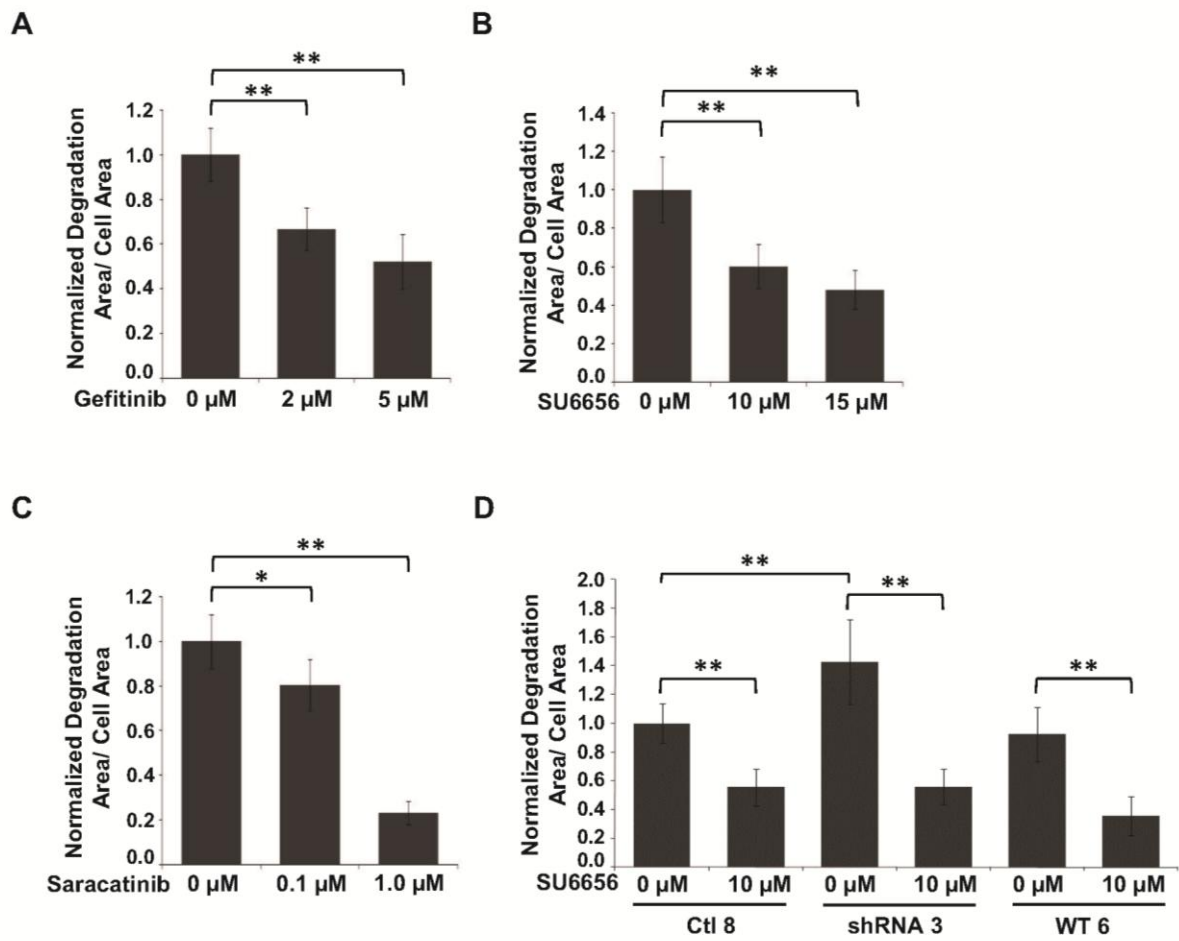




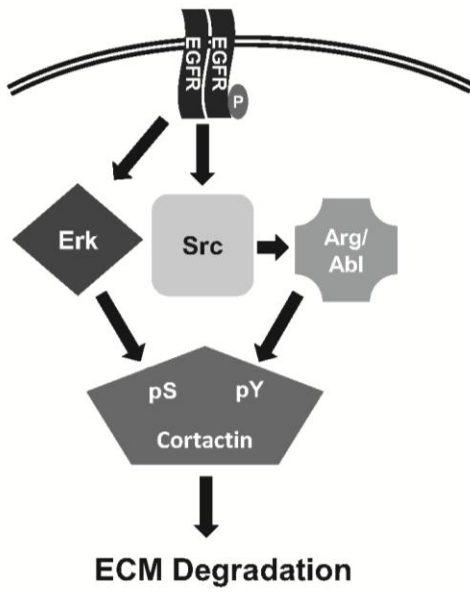
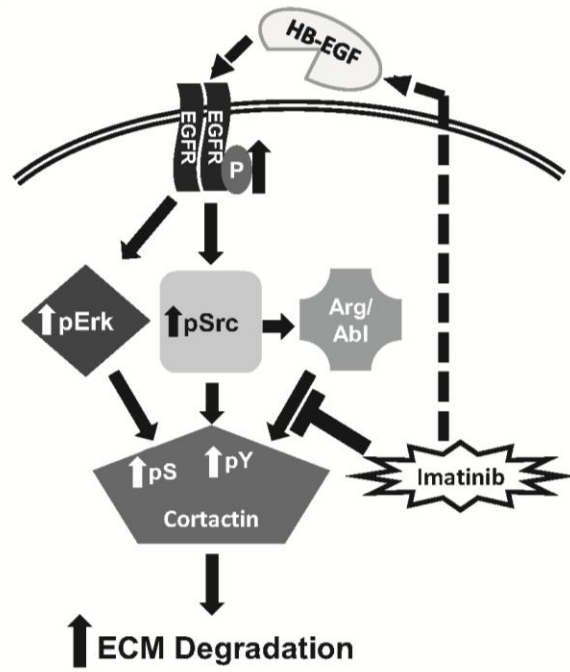
Hayes et al. Figure 5



Hayes et al. Figure 6



Hayes et al. Figure 7

**A****B**

Hayes et al. Figure 8

## **Supplementary Figure Legends**

**Supplementary Figure 1** Abl knockdown does not impact the number of HNSCC invadopodia. **(a)** Src transformed 1483 cells transiently expressing empty vector (HA-Ctl) or HA-tagged Abl (HA-Abl) are plated on Oregon Green 488-gelatin coated coverslips (pseudocolored white) for 24 h, fixed and immunolabeled with anti-HA (blue), anti-cortactin (green), and rhodamine-phalloidin (F-Actin; red). Insets demonstrate areas of overlapping invadopodia marker localization with areas of gelatin clearing. Scale bars: 10  $\mu$ m. Quantification of Abl knockdown on percentage of UMSSC1 cells degrading gelatin matrix **(b)** and invadopodia number per cell **(c)** from the assays conducted in Figure 1c. Data are represented as mean  $\pm$  C.I.

**Supplementary Figure 2** Abl knockdown does not impact matrix degradation in MDA-MB-231 cells. **(a)** MDA-MB-231 cells that were non-infected (NI) or infected with lentivirus containing control shRNA (Ctl), Abl specific (shRNA) or Abl shRNA with WT Abl re-expressed (WT) were plated on Oregon Green 488-gelatin coated coverslips (pseudocolored white) for 24h, fixed and labeled with anti-HA (blue), anti-cortactin (green) and rhodamine-phalloidin (F-Actin; red). Scale bars: 10  $\mu$ m. **(b)** MDA-MB-231 cells infected as in **(a)** were lysed and 100  $\mu$ g of cell lysate analyzed for Abl expression of by Western blot analysis. Levels of  $\beta$ -actin were evaluated to verify equal protein loading. **(c)** Quantification of Oregon Green 488-gelatin degradation from MDA-MB-231 cells transfected as in **(a)**. Error bars indicate mean percentages of gelatin degradation  $\pm$  C.I.

**Supplementary Figure 3** Determination of PDGFR $\alpha$  expression levels in OSC19, UMSSC1 and MDA-MB-231 cells. Total cell lysate (150  $\mu$ g) from each indicated line was assayed for PDGFR $\alpha$  expression by immunoblotting with anti-PDGFR $\alpha$  antibodies. NIH3T3 fibroblast lysate was included as a positive control.  $\beta$ -actin levels were assayed to verify equal protein loading.

**Supplementary Figure 4** Abl and Arg expression levels in wild-type (WT) and Abl<sup>-/-</sup>/Arg<sup>-/-</sup> (DKO) mouse embryo fibroblasts (MEFs). **(a)** Western blot analysis of Abl and Arg protein levels in WT and DKO MEFs with anti-Abl and -Arg antibodies.  $\beta$ -actin

levels were evaluated to validate equivalent protein loading. **(b)** DKO cells expressing GFP-tagged empty vector (Ctl), wild-type (WT), 527F, kinase inactive (K295M) Src, co-expressing 527F Src and wild-type (WT) Abl or wild-type (WT) MEFs expressing 527F Src were plated on Oregon Green 488-gelatin and assayed for invadopodia formation and matrix degradation for 12 h. Cells were fixed and imaged by confocal microscopy for anti-GFP (3E6; blue) or anti-Abl (yellow), and co-labelled with anti-cortactin (green) and rhodamine-phalloidin (F-Actin; red). Boxed regions and insets denote comparable regions of invadopodia formation and gelatin degradation. Scale bars: 10  $\mu$ m.

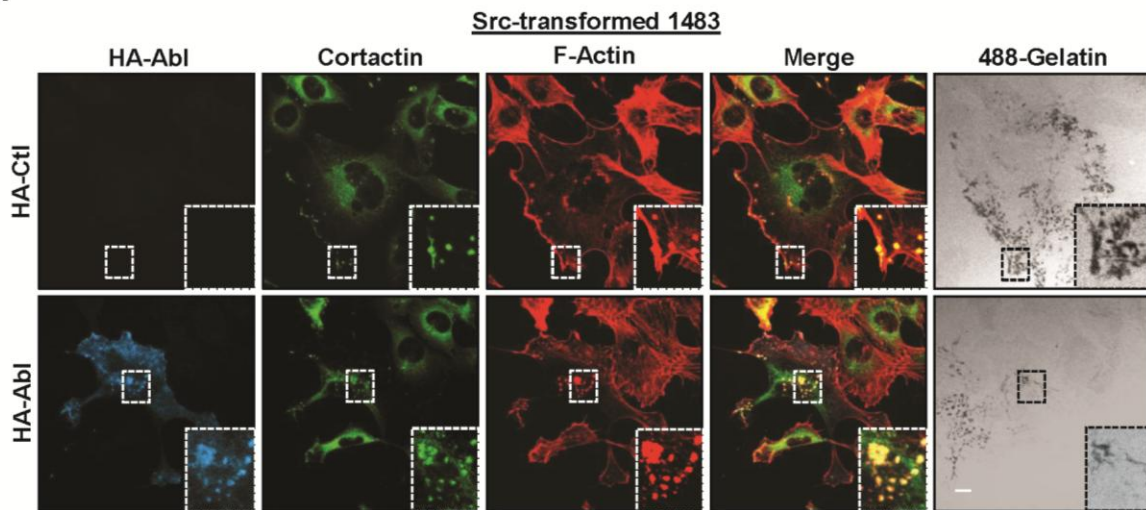
**Supplementary Figure 5** Evaluation of imatinib on EGFR and Crk signaling. **(a)** Prolonged Western exposure of the EGFR pY1068 signal from the corresponding representative panel in Figure 5a showing EGFR activation. **(b)** UMSCC1 cells were treated with indicated concentration of DMSO (0  $\mu$ M), SU6656 and/or STI571 for 24 h, lysed and assayed by Western blot analysis for Crk Y221 phosphorylation. Ratios of phosphorylated to total Crk levels are displayed. Parallel blots were probed for  $\beta$ -actin to confirm equal protein loading.

**Supplementary Figure 6** Targeted inhibition of the EGFR-Src pathway. UMSCC1 cells treated with the indicated concentrations of gefitinib **(a)**, SU6656 **(b)** and saracatinib **(c)** for 24 h were lysed and 25-50  $\mu$ g total protein analyzed for drug efficacy by Western blot analysis with the indicated phosphorylation specific antibodies. **(d)** Western blot analysis of Src inhibition in UMSCC1 cells with manipulated Abl expression. Cell lysates (50  $\mu$ g) from UMSCC1 control (Ctl 8), shRNA Abl knockdown (shRNA 3) and Abl rescued shRNA cells (WT 6) treated with DMSO (0  $\mu$ M) or SU6656 (10  $\mu$ M) for 24 h were immunoblotted with anti-pY418Src and anti-Src. Parallel blots were probed for  $\beta$ -actin to confirm equal protein loading across all lines. Ratios of phosphorylated to total Src levels are shown.

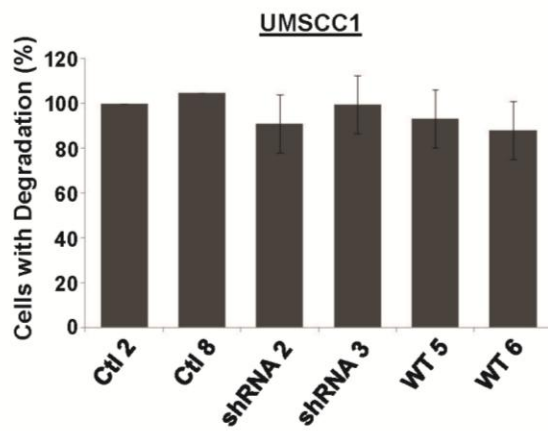
**Supplementary Figure 7** Saracatinib impairs invadopodia activity in HNSCC and MDA-MB-231 cells. Western blot analysis of 25-50  $\mu$ g of protein from **(a)** OSC19 and **(b)** MDA-MB-231 cells treated with DMSO (0  $\mu$ M) or the indicated concentrations of saracatinib for 24 h. Lysates were immunoblotted for activation of EGFR (pY1068 EGFR), Src (pY418 Src) and Abl family kinases (pY221 Crk). Parallel blots were

probed with antibodies against the non-phosphorylated protein forms as indicated. Ratios of phosphorylated/total protein relative to control for each analyzed protein and treatment condition are shown. **(c)** Effects of saracatinib on invadopodia activity. OSC19, UMSCC1 and MDA-MB-231 cells plated on Oregon Green 488-gelatin were treated with either DMSO (0  $\mu$ M) or the indicated saracatinib concentrations for 24 h. Cells were fixed and labeled with rhodamine-phalloidin and anti-cortactin antibodies. Cells and gelatin matrix were and imaged by confocal microscopy. The percentage of gelatin degradation per cell area calculated relative to controls. Data are represented as mean  $\pm$  C.I.; \*\*  $P \leq 0.01$ ; \* $P \leq 0.05$ .

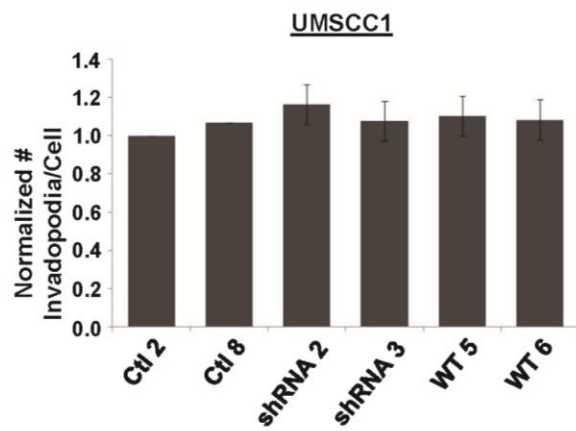
**A**



**B**

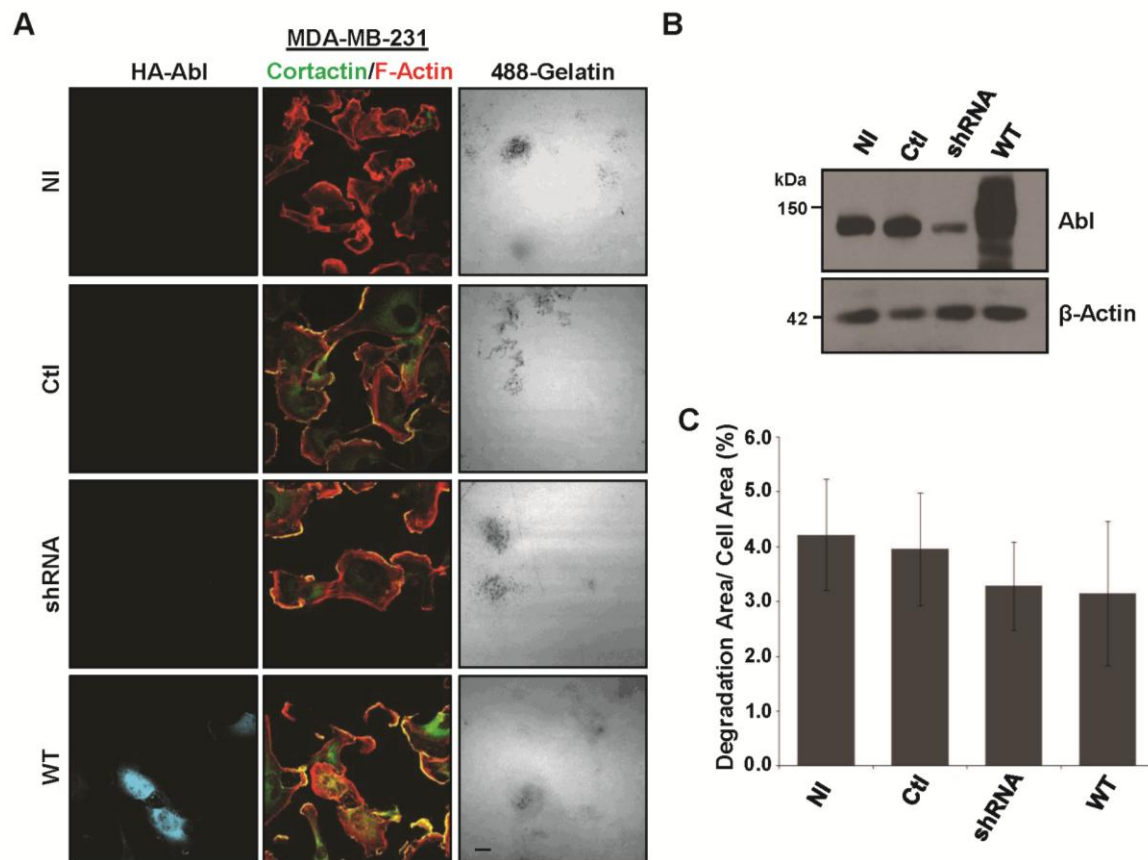


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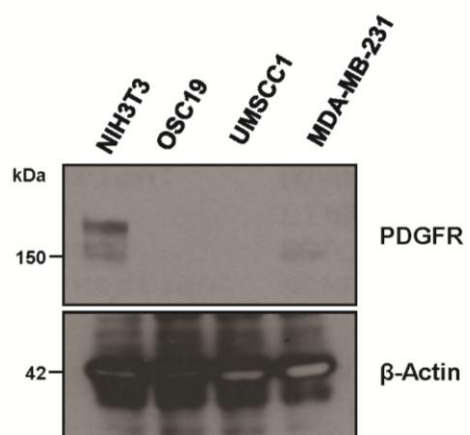


Hayes et al. Supplementary Figure 1



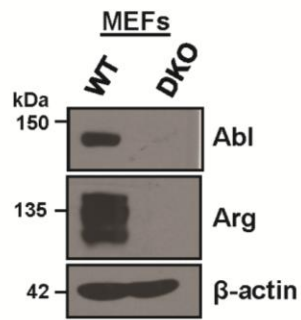


Hayes et al. Supplementary Figure 2

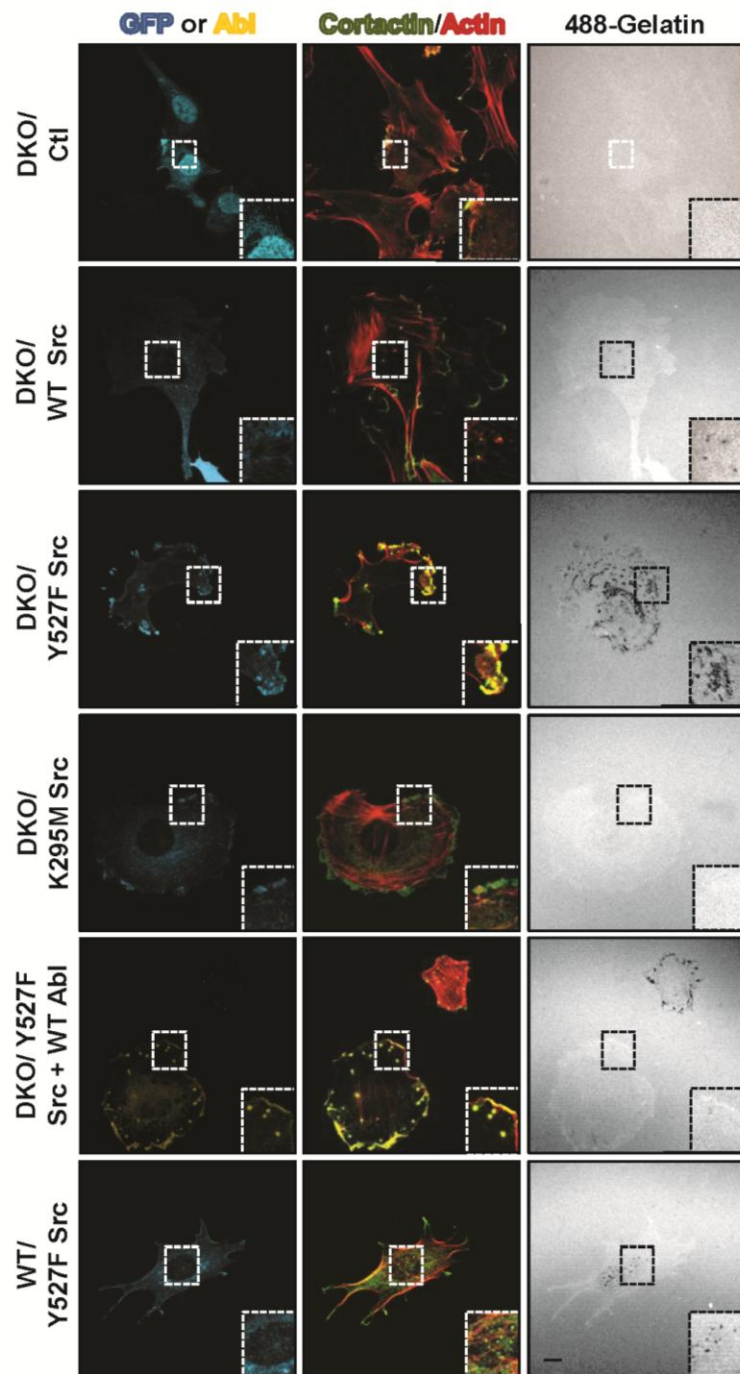


Hayes et al. Supplementary Figure 3

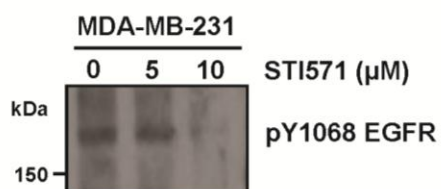
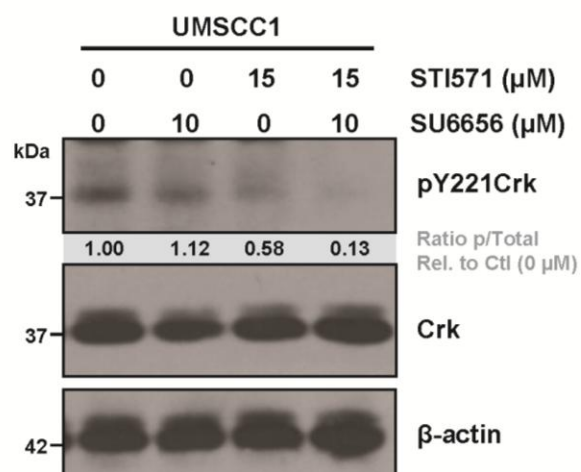
**A**



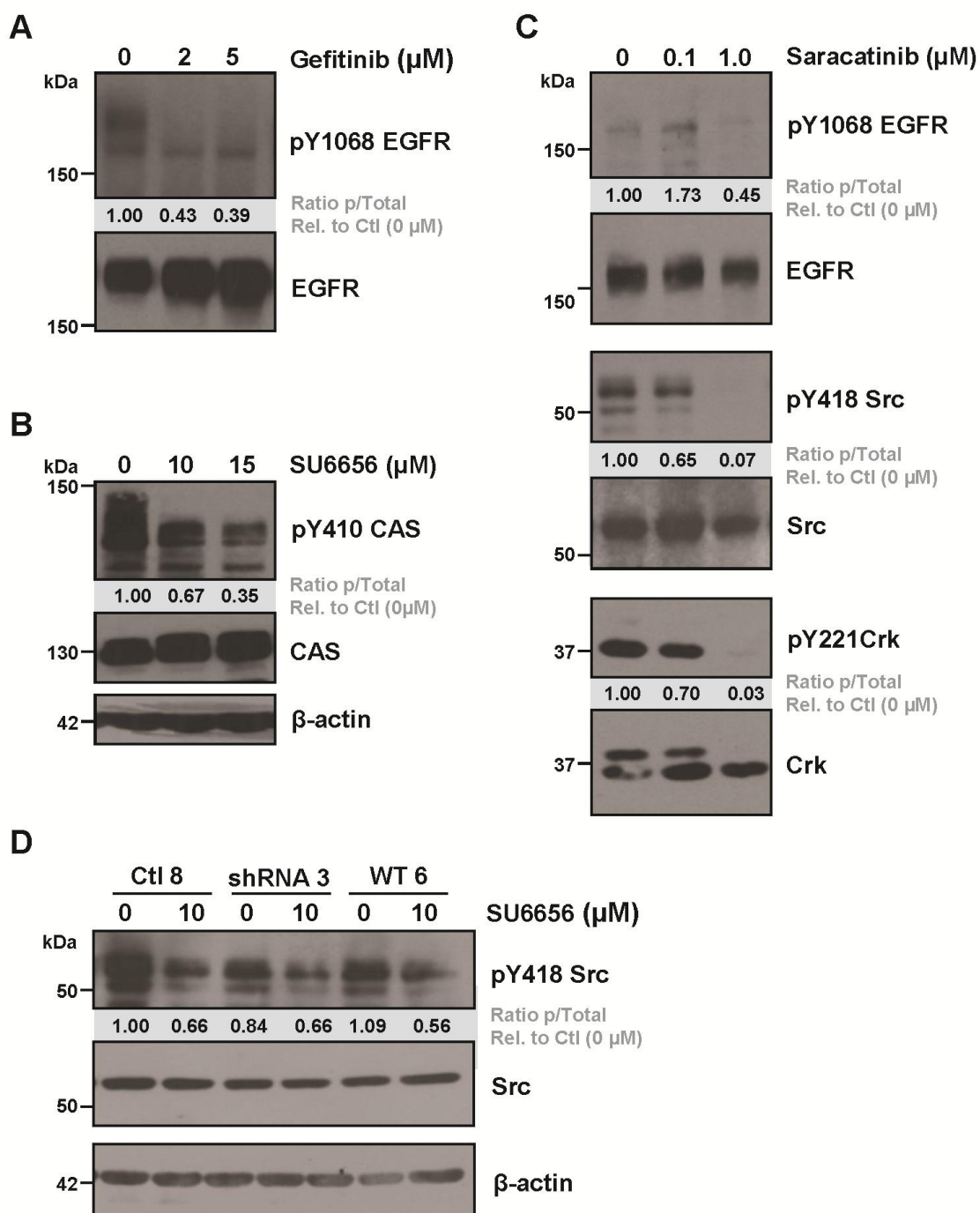
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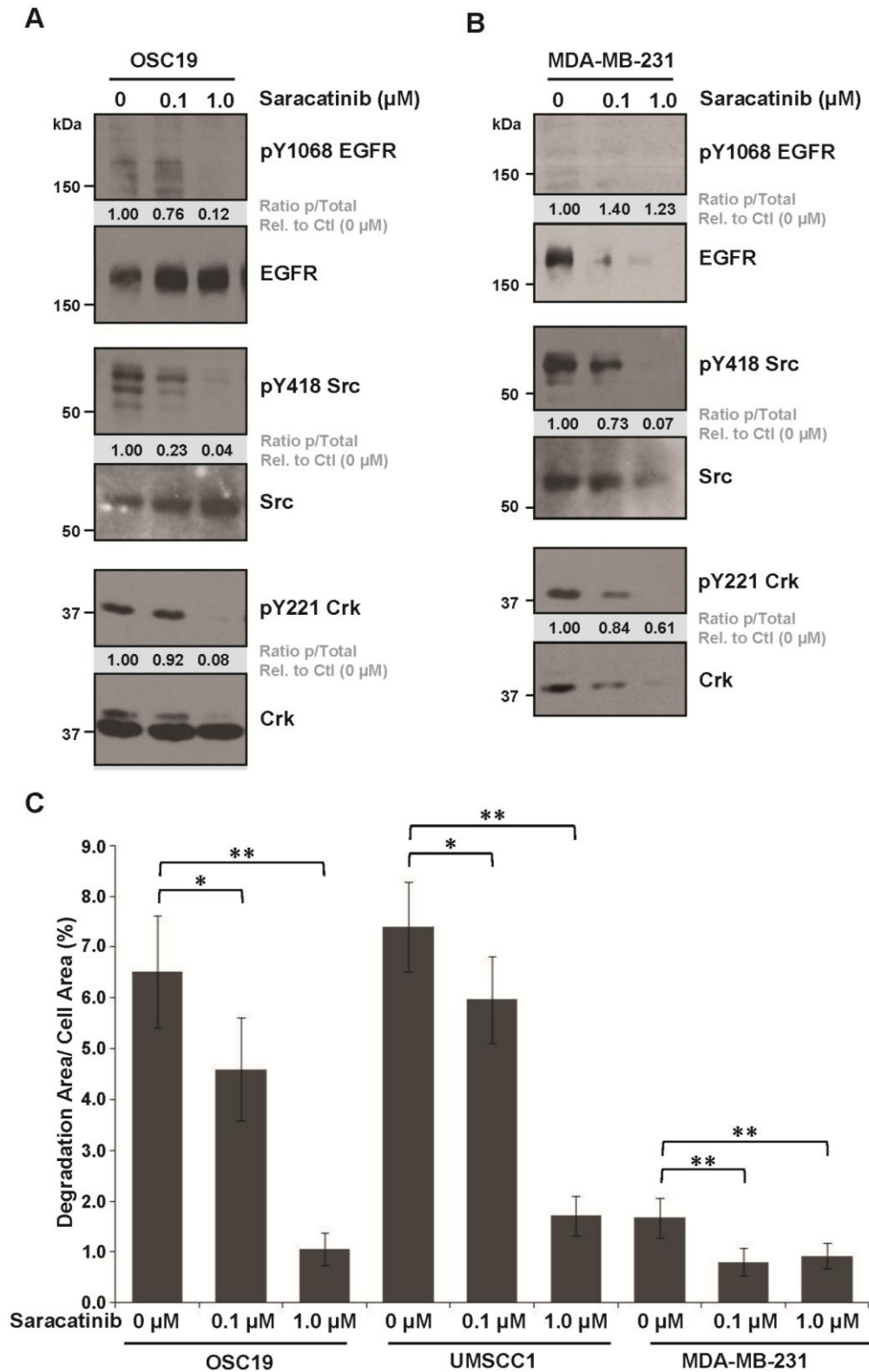
Hayes et al. Supplementary Figure 4

**A****B**

Hayes et al. Supplementary Figure 5



Hayes et al. Supplementary Figure 6



Hayes et al. Supplementary Figure 7

## **Study 2: Saracatinib Impairs Head and Neck Squamous Cell Carcinoma Invasion by Disrupting Invadopodia Function**

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Running title: Saracatinib inhibits invadopodia activity

## **Abstract**

Elevated Src kinase activity is linked to the progression of solid tumors, including head and neck squamous cell carcinoma (HNSCC). Src regulates HNSCC proliferation and tumor invasion, with the Src-targeted small molecule inhibitor saracatinib displaying potent anti-invasive effects in preclinical studies. However, the pro-invasive cellular mechanism(s) perturbed by saracatinib are unclear. The anti-proliferative and anti-invasive effects of saracatinib on HNSCC cell lines were therefore investigated in preclinical cell and mouse model systems. Saracatinib treatment inhibited growth, cell cycle progression and transwell Matrigel invasion in HNSCC cell lines. Dose-dependent decreases in Src activation and phosphorylation of the invasion-associated substrates focal adhesion kinase, p130 CAS and cortactin were also observed. While saracatinib did not significantly impact HNSCC tumor growth in a mouse orthotopic model of tongue squamous cell carcinoma, impaired perineural invasion and cervical lymph node metastasis was observed. Accordingly, saracatinib treatment displayed a dose-dependent inhibitory effect on invadopodia formation, extracellular matrix degradation and matrix metalloprotease 9 activation. These results suggest that inhibition of Src kinase by saracatinib impairs the pro-invasive activity of HNSCC by inhibiting Src substrate phosphorylation important for invadopodia formation and associated matrix metalloprotease activity.

Keywords: Saracatinib, Src, Head and Neck cancer, invadopodia, invasion, MMP



## **Abbreviations**

CAS- Crk-associated substrate

c-Src- cellular Src kinase

ECM- extracellular matrix

FACS- fluorescence-activated cell sorting

FAK- focal adhesion kinase

FITC- fluorescein isothiocyanate

HNSCC- head and neck squamous cell carcinoma

IHC- immunohistochemistry

SFK- Src family kinase

## **Introduction**

Tumor cell invasion and metastasis is a compounding problem in cancer management, with therapeutic intervention of tumor invasion becoming recognized as an increasingly relevant clinical factor (Dolgin, 2009). Increased activation of the proto-oncogene c-Src (Src) has been established in enhancing tumor progression in human cancer and corresponds with poor clinical outcome (Irby & Yeatman, 2000; Yeatman, 2004). Src is responsible for governing signaling pathways that regulate proliferation, angiogenesis, resistance to apoptosis, adhesion, motility and invasion (Summy & Gallick, 2006). High Src expression and/or activity is observed in metastases, supporting a role for Src in tumor progression by enhancing tumor invasion and metastatic potential (Summy & Gallick, 2003; Yeatman, 2004). Small molecules targeting Src kinase activity suppress proliferation, invasion and metastasis in preclinical settings (Summy & Gallick, 2006), and are currently being evaluated in clinical trials (Kopetz et al., 2007).

Head and neck squamous cell carcinoma (HNSCC) is highly invasive, frequently metastasizing to cervical lymph nodes and corresponds with poor prognosis (Kramer et al., 2005). Src overexpression is common in HNSCC (van Oijen et al., 1998) and is activated following engagement of the epidermal growth factor receptor (EGFR), where it modulates HNSCC growth and invasion through several signaling pathways (Zhang et al., 2004). The small molecule Src kinase inhibitor dasatinib suppresses motility and invasion of HNSCC cells *in vitro* and in mouse xenografts models, corresponding with decreased Src activation and invasion-associated substrate phosphorylation (Johnson et al., 2005; Sen et al., 2009). Amplification and/or overexpression of Src substrates in HNSCC correlates with poor clinical outcome, potentially serving to magnify Src pathway effects on HNSCC invasion and metastasis (Kelley et al., 2008).

HNSCC invasion and metastatic spread is mediated in part by the action of matrix metalloproteases (MMPs), with MMP1, MMP2, MMP9 and MT1-MMP activity associated with poor outcome (Rosenthal & Matrisian, 2006). MT1-MMP, MMP2 and MMP9 localize to invadopodia, actin-based ventral protrusions in invasive tumor cells that mediate focalized proteolysis of the extracellular matrix (ECM) (Linder, 2007; Weaver, 2006). Invadopodia formation is dependent on Src activity, which enhances

MMP2 and MMP9 secretion (Hsia et al., 2003; Mueller et al., 1992), and matrix degradation in HNSCC cells (Clark et al., 2007). The collective localization and action of MMPs at invadopodia allows matrix remodeling to accommodate primary tumor growth and to allow dissemination of encapsulated tumor cells to local and distant sites (Gimona et al., 2008).

Saracatinib (AZD0530) is a recently developed anilinoquinazoline inhibitor designed to disrupt Src kinase activity (Hennequin et al., 2006; Summy & Gallick, 2006). Saracatinib exhibits inhibitory effects on tumor growth in some model systems (Herynk et al., 2006), but several preclinical reports suggest that the primary anticancer effects of saracatinib are impaired tumor cell migration and invasion in HNSCC and other cancer types (Green et al., 2009; Koppikar et al., 2008; Nozawa et al., 2008). The anti-invasive effects of saracatinib are consistent with the effects of Src kinase inhibition in HNSCC by dasatinib, another Src-targeted inhibitor (Johnson et al., 2005). Saracatinib is currently being evaluated in phase I/II clinical trials for efficacy against advanced stage HNSCC and other tumor types (Kopetz et al., 2007).

Although saracatinib and other Src inhibitors are effective anti-invasive compounds, a complete understanding of the how therapeutic Src inhibition perturbs tumor invasion at the cellular level is lacking. We show that saracatinib inhibited Src activation and phosphorylation of the invadopodia regulatory proteins focal adhesion kinase (FAK), p130 Crk-associated substrate (CAS) and cortactin in HNSCC cells. Saracatinib suppressed HNSCC growth and cell cycle progression in a subset of HNSCC cell lines. Administration of saracatinib to nude mice containing orthotopic HNSCC tongue tumors inhibited Src activity, cortactin phosphorylation, perineural invasion and lymph node metastasis. We also demonstrated that saracatinib prevented invadopodia formation and ECM degradation in invasive HNSCC cells, as well as secretion and activation of MMP9. Collectively these results suggest that saracatinib exhibits anti-tumor effects in HNSCC by inhibiting invasion through the prevention of invadopodia formation. The ability of saracatinib to prevent invadopodia-mediated ECM proteolysis reveals a cellular process perturbed by Src inhibitors that is likely utilized in the progression of HNSCC and other invasive carcinomas containing high Src activity.

## **Materials and Methods**

### **Cell lines, antibodies and Western blotting**

HNSCC cell lines 1483, HN31, UMSCC 1, UMSCC19 and MSK 921 were maintained as described (Rothschild et al., 2006). Western blotting of cell lysates was conducted essentially as before (Rothschild et al., 2006). Western blotting of secreted MMP2 and 9 was conducted on conditioned media, with volumes adjusted to compensate for variations in cell numbers using dimethyl sulfoxide (DMSO)-treated cell numbers as controls.

Antibodies for immunoblotting included anti-Src (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pY418 Src (1:1000; Invitrogen, Carlsbad, CA, USA), anti-p130CAS (1:1000; BD Biosciences, San Jose, CA, USA), anti-pY410 p130CAS (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-FAK (1:1000; BD Biosciences), anti-pY861 FAK (1:1000; Invitrogen) and anti-cortactin (1 $\mu$ g/ml; 4F11 (Rothschild et al., 2006)). For detection of human pY421 cortactin, a custom antibody was developed by 21st Century Biochemicals (Marlboro, MA, USA). Briefly, a synthetic cortactin peptide encompassing the sequence around tyrosine 421 (NH<sub>2</sub>-VpYEDAASF<sub>KL</sub>-COOH) was synthesized, phosphorylated and injected into rabbits. Immune serum was passed through a column containing agarose beads coupled to the equivalent non-phosphorylated peptide, and then passed over a second agarose column containing a partially overlapping phosphorylated peptide (NH<sub>2</sub>-LPSSPVpYEDAA-COOH). Bound antibodies were eluted, concentrated and screened for specificity by Western blotting against recombinant cortactin mutant proteins harboring phenylalanine-tyrosine point mutations at tyrosine 421 (Fig. 1, Supplemental Material). Anti-ERK1/2 (Cell Signaling) and anti-pT202/pY204 ERK1/2 (Cell Signaling) were used at 1:1000. Anti-MMP2 (1:500; Millipore, Billerica, MA, USA) was used to detect cellular MMP2 levels. Secreted MMP2 was detected with antibody CA-4001 (1:100; Millipore). Cellular and secreted MMP9 was detected with monoclonal antibody 9D4.2 (1:100; Millipore). All Western blots were quantified by densitometry and ImageJ analysis, and band intensities determined relative to non-treated controls.

## **Cell proliferation and cell cycle progression assays**

For cell proliferation assays, 4,000 cells were seeded overnight and treated with 0-10  $\mu$ M saracatinib (AstraZeneca, Cheshire, UK) for 5d. 100  $\mu$ g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) was added to each saracatinib treatment condition for 4h, cells were washed and the reduced dye extracted with a 75% isopropanol/2% HCl/23% H<sub>2</sub>O mixture. Dye absorbance was read at 490 nm with an automated plate reader.

Cell cycle distribution was determined by fluorescence activated cell sorting (FACS) as previously described (Frederick et al., 2007).

## **Invasion assays**

BioCoat Matrigel invasion chambers (BD Biosciences) were rehydrated with serum-free DMEM media for 2 h.  $1 \times 10^5$  cells suspended in serum-free media were plated in the chamber insert and incubated for 2 h to allow attachment. The media in the upper and lower chambers was replaced, with serum-free DMEM added to the upper chamber and DMEM containing 5% FBS added to the lower chamber to generate a chemotactic gradient. Increasing doses of saracatinib (0-1  $\mu$ M) of saracatinib as indicated (Fig. 1D) to the upper and lower chambers. Cells were allowed to invade for 12-24 h (depending on cell line), fixed with 10% buffered formalin phosphate (Fisher Scientific, Hanover Park, IL, USA) and rinsed with PBS. Non-invasive cells were removed from the interior of the chamber insert with a swab and the remaining cells were stained with 0.4% Crystal Violet solution (Fisher) for 15 min. Invasion was assessed by counting cells in four random 20X microscopic fields.

## **Orthotopic xenograft assay of HNSCC invasion**

An *in vivo* mouse model of oral tongue squamous cell carcinoma was established as described (Myers et al., 2002) with minor modification using female athymic Foxn1<sup>nu/nu</sup> mice 4-5 weeks of age (Harlan Laboratories, Indianapolis, IN, USA). All animal procedures were conducted according to an approved protocol by the West Virginia University Animal Care and Use Committee. Anesthetized mice were injected with 2.5 x

$10^4$  UMSCC1 cells suspended in DMEM into the anterior ~1/3 of the tongue. After 10 d, treatment was initiated by daily oral gavage of 25mg/kg saracatinib suspended in a sterile solution of 0.5% methyl cellulose/0.1% polysorbate 80 (Tween 80, Sigma-Aldrich). Control animals were gavaged with the methylcellulose/Tween 80 vehicle. No overt difficulties were encountered when gavaging mice over time as the tumor size increased. Six mice were used for each treatment group. After 30 d of treatment, mice were euthanized by carbon dioxide inhalation and tumor volumes determined as described (Huang et al., 2002). Tongues, sublingual tissue containing the superficial cervical lymph nodes and the tracheoesophageal region, deep cervical and mediastinal lymph nodes, liver and lung were removed from each animal, rinsed, fixed and paraffin embedded for routine histological evaluation.

### **Immunohistochemistry**

Human HNSCC cases were obtained from the West Virginia Tissue Bank and used under the approval of West Virginia University Institutional Review Board. Five-micrometer sections from human HNSCC and mouse tissue blocks were processed and immunolabeled or hematoxylin and eosin (H&E) stained using a Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA). For immunohistochemistry, primary antibody conditions were: anti-total Src (Cell Signaling) 1:600 in Dako diluent (Dako, Carpinteria, CA, USA) for 1 h, anti-pY416 Src family kinase (SFK) (Cell Signaling) 1:25 in phosphate saline solution (PSS) (Ventana) for 12 h, anti-total cortactin (Novus Biologicals, Littleton, CO, USA) 1:700 in PSS for 1 h, anti-pY421 cortactin 1:50 in Tris-buffered saline containing 4% BSA for 1 h, and prediluted anti-cytokeratin 14 (Abcam, Cambridge, MA, USA) for 20 min. Primary antibodies were detected using the Omnimap antibody horseradish peroxidase kit (Ventana) and slides were counterstained with hematoxylin. Images were acquired as described (Rothschild et al., 2006). For quantifying pY416 Src and pY421 cortactin staining intensities, brightfield images from at least 5 randomly selected images were captured on an Olympus ZX70 Provis microscope (Olympus, Center Valley, PA, USA) with a 20x/0.70 UPlanApo objective and an Optronics MicroFire 1600x1200 color CCD camera (Optronics Inc, Goleta, CA, USA) using the StereoInvestigator imaging package

(MBF Bioscience, Williston, VT) with the same camera settings and brightfield correction enabled to ensure even illumination across the image. Brown 3,3'-diaminobenzidine (DAB) staining was separated from blue hematoxylin staining using the color deconvolution plug-in function of ImageJ (NIH) as described (Park et al., 2008). The vector values for the DAB staining were determined from ROIs with brown staining ( $R=0.425$ ,  $G=0.600$  and  $B=0.677$  for pY421 cortactin;  $R=0.475$ ,  $G=0.653$  and  $B=0.686$  for pY416 Src). Brown images were inverted and intensities were measured inside the tumor tissue. The mean DAB intensities were averaged within the group to calculate ratios of phosphorylation-specific staining in treated vs. control tissues.

### **Immunofluorescence labeling, confocal microscopy and image analysis**

UMSCC1 cells were plated on fluorescein isothiocyanate (FITC)-gelatin (Sigma) coated coverslips as described (Artym et al., 2006) for 2 h. Cells were left untreated or treated with saracatinib for 6 h, rinsed and fixed with 4% formaldehyde. Cells were permeabilized with 0.4% Triton-X/PBS for 4 minutes, then blocked in 5% BSA/PBS for 1 h. To identify invadopodia, cells were incubated with rhodamine-conjugated phalloidin (1:1000; Invitrogen), anti-cortactin monoclonal antibody 4F11 (1  $\mu\text{g/ml}$ ) and polyclonal pTyr-100 (1:200; BD Biosciences) in 5% BSA/PBS for 1 h. After washing, cells were incubated in 5% BSA/PBS containing AlexaFluor 405 goat anti-rabbit and AlexaFluor 647 goat anti-mouse secondary antibodies (Invitrogen) at 1:1000. Cells were rinsed and mounted in Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

For quantifying saracatinib effects on invadopodia incidence and matrix degradation, eight-bit 1024x1024 pixel confocal images were acquired with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss MicroImaging, Thronwood, NY, USA). Images were scanned with a 63x/1.4 NA Oil Plan-Apochromat objective at 1.3x zoom, yielding a resolution of 10.14 pixels/ $\mu\text{m}$ . All images of the FITC-gelatin were taken with the same parameters (pinhole size, laser intensity and gain) so image intensity would be comparable between samples. For invadopodia formation, a minimum of six independent fields comprising > 50 cells were analyzed for cells containing invadopodia compared to total cell number. For matrix degradation, cells were analyzed using ImageJ software. Actin images were adjusted to threshold values to include all cellular

regions, and the resulting images were used to calculate total cell areas in  $\mu\text{m}^2$ . For quantifying matrix degradation, FITC-gelatin images were inverted so that regions with increased degradation would yield higher intensity values, ensuring selection of all areas of matrix degradation. The integrated density was reported as the amount of degradation per total cell area. A minimum of 15 cells was analyzed for each saracatinib concentration.

### **Gelatin zymography**

UMSCC1 and 1483 cells were plated overnight at  $5 \times 10^6$  in complete media and were treated with saracatinib at increasing dosage for 24 h, rinsed and incubated for 24 h in serum-free media containing the equivalent saracatinib dose. Cells were counted, and conditioned media collected and concentrated by ultrafiltration using Amicon Ultra-4 centrifugal filter devices with a 10kDa molecular weight cutoff (Millipore). Zymography was conducted as described (Clark et al., 2007) with minor modification. Conditioned media (35  $\mu\text{l}$ ) was diluted in 2X non-reducing SDS-PAGE sample buffer and resolved on 8% SDS-PAGE gels containing 1 mg/ml bovine gelatin (Sigma). Aliquots of serum-free DMEM and DMEM containing 10% FBS were used as negative and positive controls, respectively. MMP activity was renatured by washing gels in 2.5% Triton X-100 for 30 min, followed by washing gels in Developing Buffer (50 mM Tris, 0.2M NaCl, 5mM  $\text{CaCl}_2$ , 0.02% Brij 35) for 30 min at room temperature. Gels were then incubated in renewed Developing buffer for 24h at 37° C to allow MMP activity to proceed. Gels were stained with Coomassie Brilliant Blue R-250 (0.5% w/v) for 30 min, followed by destaining in water. Resulting gels were scanned using a FotoAnalyst Investigator (Fotodyne Inc, Hartland, WI, USA) and areas of gelatinase activity quantified using ImageJ. Results were adjusted relative to control DMSO treatment for gelatinase activity and cell counts for each treatment to compensate for variations in final cell numbers.

### **Statistical analysis**

Differences in mean values between saracatinib treatment groups for invasion, invadopodia and gelatinase assays were evaluated using one-way ANOVA, followed by



Student-Newman-Keuls post hoc testing. Differences were considered significant at  $P < 0.05$ , with all experimentation conducted at least in triplicate.

## **Results**

### **Saracatinib effects on HNSCC proliferation and invasion**

As a first step in our work, we characterized several HNSCC cell lines previously determined to have varying degrees of invasive and metastatic potential (Rothschild et al., 2006; Sano & Myers, 2007; Yang et al., 2004) for their response to saracatinib. To determine the effect of saracatinib treatment on proliferation in these lines, growth inhibition was assessed by 5d 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays for cells treated with increasing doses of saracatinib (Fig. 1A). The HN31 and UMSCC1 lines were sensitive to growth inhibition by saracatinib, as indicated by sub-micromolar  $IC_{50}$  values (Fig. 1A). In contrast, the 1483 line demonstrated a marked resistance to saracatinib, with an  $IC_{50} = 7.60\mu M$ . Corresponding effects on cell cycle progression are also observed, with increasing saracatinib concentrations resulting in enhanced  $G_1$  checkpoint arrest in HN31 and UMSCC1 lines determined by FACS analysis (Fig. 1B). Saracatinib treatment did not alter the percentage of cells undergoing  $G_2$ -M transition for either line. Arrest of  $G_1$  was not evident in 1483 cells treated with saracatinib concentrations up to  $1\mu M$ , in agreement with resistance of this line in proliferation analysis (Fig. 1A). Erk1/2 activation, a potent driver of mitogenesis, was impaired in HN31 and UMSCC1 cells at concentrations above  $0.5\mu M$  as determined by Western blotting (Fig. 1C). Similar results were obtained for the invasive HNSCC lines MSK921 and UMSCC19 (data not shown). Erk1/2 activation was elevated in 1483 cells at doses up to  $1\mu M$  (Fig. 1C), in agreement with the high  $IC_{50}$  value for this line (Fig. 1A). These data indicate that the HNSCC lines used in this study vary in their proliferative response to saracatinib treatment, and can be segregated into sensitive (HN31 and UMSCC1) and resistant (1483) populations.

Next we evaluated the effect of saracatinib on HNSCC invasion *in vitro* using modified Boyden chamber transwell assays. In the absence of saracatinib, UMSCC1 and HN31 cells displayed an invasive response to serum, averaging 4150 cells/aggregate field and 1719 cells/aggregate field, respectively (Fig. 1D). 1483 cells were weakly invasive, with an average of 243 cells/aggregate field (Fig. 1D). Increased concentrations of saracatinib resulted in dose-dependent inhibition of HNSCC invasion for all tested lines,

with reduced invasion compared to control levels of 88% in UMSCC1, 70% in HN31, and 78% in 1483 cells at the highest evaluated dose (1.0  $\mu$ M) (Fig. 1D). These results indicate that saracatinib directly impacts the ability of HNSCC cell lines to invade in an *in vitro* setting.

### **Saracatinib inhibits Src activation and substrate phosphorylation in HNSCC cells**

To determine the impact of saracatinib on Src activity and phosphorylation of invadopodia-related Src substrates, dose-dependence experiments were performed on HN31, UMSCC1 and 1483 cells (Fig. 2). Cell lines were treated with increasing doses of saracatinib for 24 hours, lysed and assessed for phosphorylation by immunoblotting using anti-phosphorylation site-specific antibodies to detect Src activation (pY418) and specific Src phosphorylation sites in downstream substrates (pY410 p130 CAS, pY421 cortactin and pY861 FAK). The phosphorylation of Src at Y418 was inhibited by saracatinib in all tested lines at concentrations between 0.5 and 1.0  $\mu$ M (Fig. 2). Phosphorylation of cortactin at tyrosine 421 and FAK at tyrosine 861 was also reduced within the same range of saracatinib concentrations (FAK phosphorylation in 1483 cells could not be evaluated due to the absence of detectable FAK expression in this line). While phosphorylation of tyrosine 410 in p130 CAS was inhibited within this same dose range in UMSCC1 cells, we observed that p130 CAS phosphorylation was consistently inhibited at lower dose ranges (0.01-0.05  $\mu$ M) in HN31 and 1483 cells (Fig. 2). Immunoblotting with antibodies against total p130 CAS, cortactin and FAK indicate that the expression levels of these proteins are somewhat reduced at high dose levels of saracatinib treatment (0.5-1 $\mu$ M), but not at levels accountable for the resultant decrease of tyrosine phosphorylation at the assayed sites (0.1-0.5 $\mu$ M).

### **Saracatinib inhibits Src activation, invasion and cervical lymph node metastasis in an orthotopic mouse model of oral squamous cell carcinoma**

A mouse orthotopic model of tongue squamous cell carcinoma (Myers et al., 2002) that phenotypically mimics human HNSCC (Fig. 3) was utilized to evaluate the effects of saracatinib on HNSCC progression and invasion in an *in vivo* setting. Athymic mice with UMSCC1 tongue tumors were randomized and treated with either vehicle or daily

with 25mg/kg saracatinib. At the end of treatment (40d), mice from both groups had similar weight (24g) and mean tumor volumes ( $54.1\text{mm}^3 \pm 2.3\text{mm}$  for controls;  $43.7\text{mm}^3 \pm 4.6\text{mm}$  with saracatinib treatment). The modest impact of saracatinib on UMSCC1 tumor growth was not statistically significant. To determine the impact of saracatinib on Src activity, primary tumors were evaluated for Src and cortactin phosphorylation by immunohistochemistry. Active Src (determined by pY416 SFK staining) and pY421 cortactin labeling in UMSCC1 tumors displayed similar patterns compared to human HNSCC (Fig. 3A). Saracatinib treatment reduced the ratios of pY416 SFK and pY421 cortactin compared to control-treated mice (Figure 3A).

Human HNSCC often displays perineural invasion with regional lymph node involvement. Given the invasive nature of UMSCC1 cells (Fig. 1D), we evaluated the impact of saracatinib treatment on loco-regional tissue invasion and cervical lymph node metastasis in treated mice. Soft tissues from the submental space through the tracheoesophageal region were evaluated for perineural invasion and cervical lymph node metastasis by immunostaining for the epithelial marker cytokeratin 14. Similar to human tumors, extensive perineural invasion and metastasis to the superficial cervical lymph nodes was evident in 5/6 control treated mice (Fig. 3B). Invasion and lymph node metastasis was found in 1/6 saracatinib-treated mice, and the remaining mice displayed a complete absence of cytokeratin-positive cells associated with nerves, sublingual glands, connective tissue or cervical lymph nodes (Fig. 3B). These data demonstrate that the *in vivo* ability of saracatinib to down-regulate Src activity and cortactin phosphorylation correlates with decreased invasion and local lymph node metastasis.

### **Saracatinib inhibits invadopodia formation and matrix degradation in UMSCC1 cells**

UMSCC1 cells plated on fluorescently-labeled gelatin formed centrally localized ventral puncta enriched with cortactin, filamentous (F)-actin and phosphotyrosine (Fig. 4A, DMSO), three markers that define invadopodia (Bowden et al., 2006). Spontaneous invadopodia formation was observed in 51% of UMSCC1 cells by confocal microscopy at a given time, corresponding with focalized areas of matrix clearing (FITC-gelatin)

(Fig. 4B, 0 $\mu$ M dosage point). The gelatin matrix underneath UMSCC1 cells typically displayed degradation encompassing ~53% of the overlying cell area, reflecting the action of invadopodia-associated MMP activity (Fig. 4C).

UMSCC1 cells plated on FITC-coated gelatin were treated with increasing concentrations of saracatinib, and invadopodia formation and matrix degradation was evaluated by confocal microscopy (Fig. 4). The number of cells containing invadopodia was significantly decreased with increasing saracatinib dosage, with < 2% of cells having formed invadopodia at concentrations at or above 0.5  $\mu$ M (Fig. 4B). Effects on matrix degradation were more pronounced, where increased saracatinib dosage resulted in similar incremental decreases in degradation (Fig. 4C). UMSCC1 cells treated with 1.0  $\mu$ M saracatinib did not contain invadopodia and were incapable of degrading matrix (Fig. 4A). These cells also lacked focal cortactin localization, had diminished phosphotyrosine levels at focal adhesions and contained disorganized F-actin puncta on the ventral membrane surface where invadopodia typically form (Fig. 4A, arrowheads).

### **Saracatinib inhibits MMP9 secretion from HNSCC cells**

In addition to MT1-MMP, secretion and activation of MMP2 and MMP9 at invadopodia has been reported to be partially responsible for the observed effects on matrix degradation (Linder, 2007). Src activity regulates MMP2 and MMP9 secretion in fibroblasts (Hsia et al., 2003). We therefore determined the effect of saracatinib on MMP secretion. Confocal immunofluorescence microscopy indicated that UMSCC1 cells have MMP9-containing vesicles localized to invadopodia at sites that correspond with gelatin degradation (Fig. 5A), indicating that MMP9 is concentrated in UMSCC1 invadopodia. To evaluate the impact of saracatinib on MMP2 and MMP9 secretion and activity in HNSCC cells, total cell lysates and conditioned media from saracatinib-treated 1483 and UMSCC1 cells were analyzed for the presence of cellular and secreted MMP2 and MMP9 by Western blotting (Fig. 5B). Saracatinib treatment resulted in modest decreases (up to 24%) in cellular MMP2 levels at concentrations to 1  $\mu$ M, while cellular MMP9 levels demonstrated up to a two-fold increase under the same concentration range. Although secreted MMP2 was not detected in the media of either

cell line, both lines secreted detectable amounts of MMP9 (Fig. 5B). Treatment of either line with saracatinib inhibited MMP9 secretion, and each line displayed differential drug sensitivity. Detectable MMP9 secretion from 1483 cells was largely absent at the lowest evaluated concentration (0.01  $\mu$ M) whereas secretion from the more invasive UMSCC1 line was inhibited at concentrations of 0.5  $\mu$ M and above (Fig. 5B). The secreted MMP9 from both lines displayed proteolytic activity when assayed by gelatin zymography (Fig. 5C). Saracatinib concentrations up to 0.1  $\mu$ M did not significantly affect MMP9 activity as determined by ANOVA analysis for both lines, although mean values for 1483 cells treated with these lower doses were consistently below control levels (Fig. 5C). Higher saracatinib concentrations (0.5  $\mu$ M and 1.0  $\mu$ M) reduced MMP9 activity to respective mean values of 39% and 25% for control levels in 1483 cells, and 22% and 12% in UMSCC1 cells (Fig. 5B,C). These data indicate that saracatinib treatment of HNSCC cells leads to selective inhibition of MMP9 secretion in 1483 and UMSCC1 cells, preventing efficient enzymatic degradation of extracellular matrix components.

## **Discussion**

The present study demonstrates that inhibition of HNSCC invasion in preclinical *in vitro* and *in vivo* settings by saracatinib directly corresponds to disruption of HNSCC invadopodia formation and function, identifying invadopodia as a potential downstream target of therapeutic Src kinase inhibition in HNSCC and other invasive human cancers. Invasive HNSCC presents a difficult problem in patient care, given the proximity of most tumors to multiple vital organ sites in the head and neck region. Disregulation of signaling pathways that promote and sustain invasion impinge on adhesion- and cytoskeletal-associated proteins. These proteins function in concert with MMPs to enable tumor cells to degrade and protrude through an encapsulating ECM, allowing movement into neighboring tissues. The ability of saracatinib to ablate invadopodia and the associated invasive behavior of HNSCC cells in mice provides further evidence for a direct link between invadopodia activity and tumor invasion, shedding light on the specific invasion-promoting cellular processes perturbed by Src kinase inhibition.

The HNSCC lines used in this study displayed differential responses to saracatinib in terms of anti-proliferate effects, with some lines (HN31 and UMSCC1) having submicromolar sensitivity, cell cycle inhibition and decreased ERK1/2 activity and others (1483) demonstrating resistance to the drug at concentrations up to 1  $\mu$ M (Fig. 1A-C). While a recent study reported IC<sub>50</sub> saracatinib values near 1  $\mu$ M for five different HNSCC lines (Koppikar et al., 2008), our findings are in line with the wider range of IC<sub>50</sub> values reported for different HNSCC lines treated with the non-related Src kinase inhibitor dasatinib (Johnson et al., 2005) as well as in other tumor cell types (Boyer et al., 2002; Johnson et al., 2005; Jones et al., 2002). These reports taken together with our data suggest that the HNSCC lines utilized in this study fall within the typical *in vitro* proliferative response profile to therapeutic Src inhibition. While the underlying compensatory mechanism for saracatinib resistance in 1483 cells is unknown, future expression profiling of resistant and sensitive lines may provide insight into the molecular nature of saracatinib resistance, as has been recently conducted for the EGFR inhibitor gefitinib in a variety of HNSCC lines (Frederick et al., 2007).

Saracatinib treatment resulted in *in vitro* anti-invasive activity, impaired Src activation and tyrosine phosphorylation of FAK and p130 CAS in all analyzed HNSCC lines (Fig. 1D; Fig. 2). This is in accord with other studies on other HNSCC lines utilizing saracatinib or dasatinib as single agents (Johnson et al., 2005; Koppikar et al., 2008; Nozawa et al., 2008). Tyrosine phosphorylation of FAK and p130 CAS have been commonly utilized as downstream indicators for preclinical therapeutic anti-Src efficacy, since Src-mediated phosphorylation of these proteins are critical events in enabling tumor invasiveness (Brabek et al., 2005; Zhao & Guan, 2009). In addition, we show that cortactin tyrosine phosphorylation was also reduced following saracatinib treatment (Fig. 2). Cortactin is a Src substrate commonly overexpressed in invasive HNSCC and regulates invadopodia formation (Rodrigo et al., 2000; Rothschild et al., 2006; Weaver, 2008). Cortactin phosphorylation is important for tumor cell motility and matrix degradation at invadopodia (Ayala et al., 2008; Oser et al., 2009; Rothschild et al., 2006). These results suggest that cortactin tyrosine phosphorylation status can serve as an additional downstream monitor of Src activity and invasive potential in HNSCC cells where Src kinase function is impaired.

While saracatinib was able to modestly decrease the size of *in vivo* UMSCC1 tongue tumors, the reduction in tumor size was not significant compared to untreated controls. UMSCC1 cell growth is inhibited by saracatinib *in vitro* (Fig. 1A), implying that microenvironmental factors such as inflammatory cytokines, growth factors, neoangiogenic and hypoxic aspects responsible for promoting and maintaining HNSCC growth (Pries & Wollenberg, 2006; Timar et al., 2005) may partially circumvent the growth-inhibitory effects of saracatinib in UMSCC1 xenografts. On the other hand, saracatinib displays *in vivo* anti-invasive properties by potently inhibiting perineural invasion and cervical lymph node metastasis. Suppressed Src activation and cortactin tyrosine phosphorylation in primary tumors (Fig. 4A) supports this conclusion, as signaling through these proteins promotes invasion and metastatic spread (Weaver, 2008; Yeatman, 2004). Recent findings in a comparable xenograft system utilizing mice treated with dasatinib demonstrated reduced Src and FAK activity (Sen et al., 2009). EGFR overexpression is common in HNSCC, resulting in enhanced Src activity, cortactin phosphorylation and tumor invasiveness (Koppikar et al., 2008; Rothschild et



al., 2006). Clinical EGFR inhibitors also display anti-invasive activity and impair invadopodia formation in preclinical settings (Huang et al., 2002; Yamaguchi et al., 2005; Yang et al., 2004), suggesting in light of our findings that inhibiting EGFR activity in HNSCC impairs Src activation and substrate phosphorylation required for invasion. This is supported by emerging rationale for dual targeting of EGFR and Src in treating advanced HNSCC (Egloff & Grandis, 2008).

Elevated Src activity is necessary and essential for invadopodia formation (Chen et al., 1985; Chen et al., 1984). The ability of saracatinib to ablate invadopodia and associated matrix degradation demonstrates that a clinically utilized Src inhibitor disrupts a vital subcellular structure required for tumor invasion. Src-induced invadopodia formation in carcinoma cells first targets cortactin and F-actin formation at matrix adhesion sites, forming a core preinvadopodia complex. Recruitment of MT1-MMP to preinvadopodia initiates matrix degradation and invadopodia maturation, with further maturation involving dissolution of the cortactin-F-actin complex, focal retention of MT1-MMP and continued proteolytic activity (Artym et al., 2006). While rudimentary invadopodia-like F-actin structures formed in UMSCC1 cells treated with inhibitory concentrations of saracatinib, they do not contain cortactin (Fig. 4A), which is essential for invadopodia formation (Artym et al., 2006), indicating that Src kinase activity is required for cortactin localization to invadopodia. Similar results have been shown through the use of kinase-inactive Src constructs (Bowden et al., 2006). Tyrosine phosphorylation of invadopodia proteins is strongly linked with the ability to degrade extracellular matrix (Bowden et al., 2006), with Src phosphorylation of cortactin (Ayala et al., 2008), paxillin (Badowski et al., 2008), and ASAP1 (Bharti et al., 2007) requisite for invadopodia formation and/or proteolytic activity. Src kinase inhibition therefore displays at least a two-fold effect on substrates in invadopodia by impairing proper preinvadopodia targeting and perturbing phosphotyrosine-based signaling dynamics involved in regulating invadopodia maturation and function. Tyrosine phosphorylation in peripheral focal adhesions was observed in UMSCC1 cells, indicating saracatinib may also perturb focal adhesion formation and/or function given the critical role for Src and related kinases in these structures (Frame, 2004).

MMP activity is essential for HNSCC invadopodia formation and function (Clark et al., 2007). The impairment of MMP9 secretion and activation from HNSCC cells treated with saracatinib indicates that Src kinase activity is required for targeting and secretion of MMP9-containing vesicles at invadopodia. This is in agreement with observed effects of Src kinase inhibition on MMP9 secretion in other tumor types (Cortes-Reynosa et al., 2008; Lee et al., 2005). The lack of apparent MMP2 secretion in the HNSCC lines used in our studies precluded evaluation of this metalloproteinase, and is likely characteristic to these lines since other HNSCC cells secrete MMP2 (Clark et al., 2007). Localization of transmembrane and secreted MMPs to invadopodia involves directed trafficking of vesicles emanating from the *trans*-Golgi network, where a dynamin-2-N-WASp-Arp2/3-cortactin complex has been implicated in coupling cortical actin regulation with invadopodia membrane dynamics (Buccione et al., 2004). Cortactin has been implicated as a key regulator of MT1-MMP surface expression and MMP2 and MMP9 secretion in HNSCC (Clark & Weaver, 2008; Clark et al., 2007). While it is currently unknown how cortactin regulates the targeting of MMP-containing vesicles, Src-mediated phosphorylation may play a vital role since tyrosine phosphorylation of cortactin increases its binding to vesicle-associated proteins (Ammer & Weed, 2008) and is required for efficient invadopodia-mediated ECM degradation (Ayala et al., 2008; Webb et al., 2007).

Our results indicate that disruption of Src activity by saracatinib impairs HNSCC cell invasion and lymph node metastasis by preventing invadopodia formation and function, identifying a cellular mechanism that may be universally impacted by Src inhibition in invasive carcinoma cells. In addition to Src and related kinases, saracatinib also inhibits Abl kinase, an activity that has been exploited to evaluate imatinib-resistant chronic myelogenous leukemia (CML) cases expressing the constitutively active BCR-Abl Philadelphia chromosome gene product (Gwanmesia et al., 2009). Along with the tumor-promoting activities in CML, elevated Abl kinase activity has been shown to be important in breast (Srinivasan & Plattner, 2006) and non small cell lung cancer (Lin et al., 2007), indicating Abl kinase activity has a functional role in solid tumor progression (Lin & Arlinghaus, 2008). Abl expression has been evaluated in oral squamous cell carcinoma and correlates with tumor stage (Yanagawa et al., 2000), suggesting that

elevated Abl expression may contribute to HNSCC progression. While a mechanism pertaining to Abl function in solid tumor invasion or invadopodia function has not been reported, Abl does bind and phosphorylate cortactin, (Boyle et al., 2007), raising the potential for Abl kinase to play a role in cortactin-based invadopodia function in HNSCC and other invasive Abl-expressing solid tumors. Such a role for Abl in regulating tumor invasion would also likely be impaired by saracatinib and other dual Src/Abl inhibitory compounds.

In addition to carcinoma invadopodia, Src-mediated processes in non-cancerous cell types involved in promoting invasion may also be impacted by Src family kinase inhibition. The motility and protease remodeling ability of tumor stromal fibroblasts involved in enabling collective HNSCC invasion could be affected by impairing Src family kinases *in vivo* (Gaggioli et al., 2007), as well as the proinvasive properties of tumor-associated macrophages (Condeelis & Pollard, 2006). The ability of saracatinib and other Src inhibitors to impair functions of different cellular types that propagate tumor invasion provides the opportunity for the future discovery of additional Src-based cellular mechanisms utilized during tumor progression.

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## **Figure Legends**

**Figure 1** Effects of saracatinib on HNSCC proliferation, cell cycle progression, Erk1/2 activation and *in vitro* invasion. **A.** IC<sub>50</sub> values for cell growth determined by 5 day MTT assays for the indicated HNSCC lines treated with 0-10  $\mu$ M saracatinib. Mean values are shown for each line from three independent assays. **B.** Impact of saracatinib on HNSCC cell cycle progression. HNSCC cell lines were treated with the indicated amounts of saracatinib for 24 hours, fixed labeled with propidium iodide to assess DNA content, and analyzed for cell cycle status by fluorescence-activated cell sorting. Results show the average percentage of cells in each cell cycle phase as indicated on the *left*. Bars, SD of two independent experiments. **C.** Effects of saracatinib on Erk1/2 activity. HNSCC cells were treated with saracatinib for 24 hours at the indicated doses, lysed and analyzed by Western blotting with phosphorylation-specific (pErk1/2) and total Erk1/2 antibodies. Blots shown are representative of three different experiments, with indicated band intensities shown relative to no treatment (0  $\mu$ M) for each cell line. **D.** Saracatinib inhibits *in vitro* HNSCC invasion. HNSCC cells ( $1 \times 10^5$ ) were plated in Matrigel-coated transwells alone or with increasing concentrations of saracatinib. After 2 h, invasion was stimulated with 5% FBS and cells were allowed to invade for 12 h (UMSCC1) or 24 h (HN31 and 1483). Invaded cells were quantified by brightfield microscopy. Bars, SEM of three independent experiments.

**Figure 2** Saracatinib inhibits Src activity and downstream Src substrate phosphorylation in HNSCC cell lines. HN31, UMSCC1 and 1483 cells were treated with DMSO vehicle or saracatinib (0.01-1  $\mu$ M) for 24 h. Cells were lysed and total protein amounts were analyzed by Western blotting with total or phosphorylation site-specific antibodies for Src and the indicated substrates. Blots shown are representative of at least four independent experiments, with band intensities for each substrate quantified relative to the untreated (0  $\mu$ M) condition for each cell line.

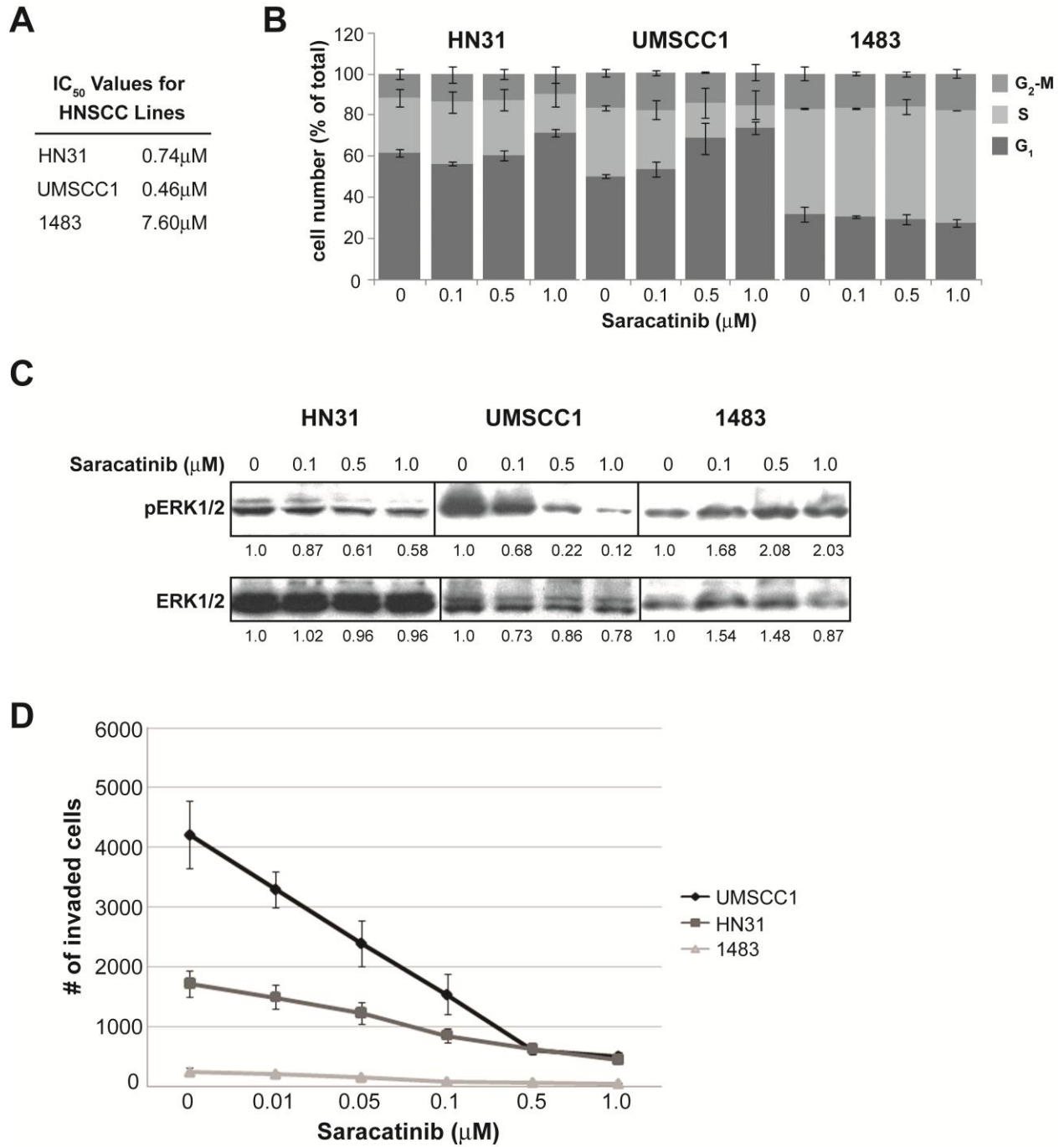
**Figure 3** Saracatinib inhibits Src activity, perineural invasion and cervical lymph node metastasis in orthotopic UMSCC1 tongue tumors. **A.** UMSCC1 tongue tumors from representative control-treated or saracatinib-treated mice were sectioned and stained with hematoxylin and eosin (H&E) or by IHC with the indicated antibodies (*left*). A case

of human HNSCC was evaluated in parallel as a positive control. The pY416 SFK and pY421 cortactin ratios from saracatinib treated to control levels are indicated. *Bars*, 100  $\mu\text{m}$ . **B.** Locoregional invasion and lymph node metastasis is inhibited by saracatinib. Submental and associated tracheoesophageal tissue from control treated and saracatinib treated mice was immunostained for cytokeratin 14 to detect cells of epithelial origin. Magnified regions containing a single sublingual nerve and superficial cervical lymph node are shown for clarity. *Inset* shows a magnified cortical region of superficial cervical lymph nodes from control and saracatinib treated mice. Arrowheads denote metastasized UMSSC1 cells. N; sublingual nerve, ED; excretory duct. *Bars* 100  $\mu\text{m}$ ; inset, 50  $\mu\text{m}$ .

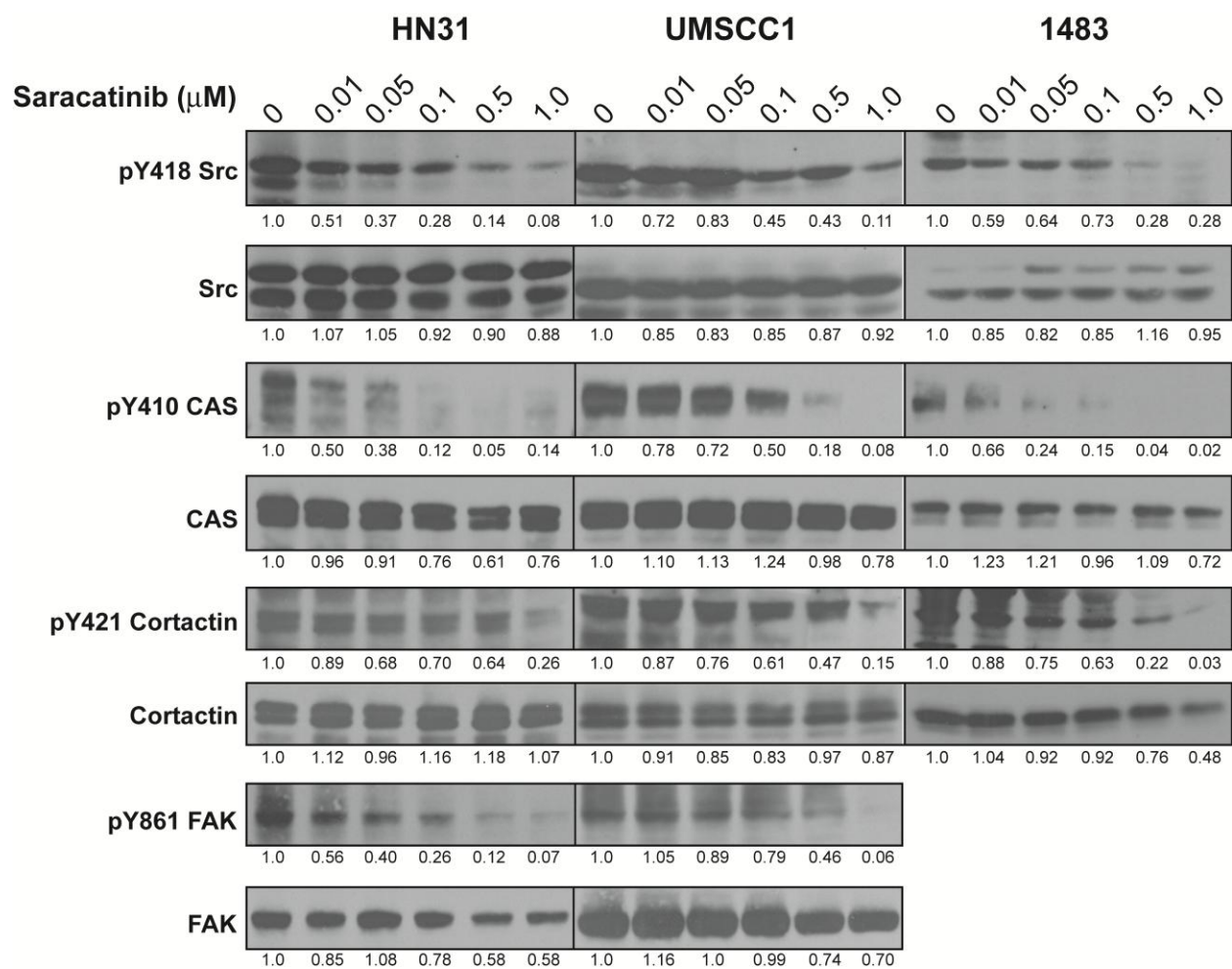
**Figure 4** Saracatinib inhibits invadopodia formation and ECM degradation. **A.** Representative images of UMSSC1 cells treated with different saracatinib concentrations. UMSSC1 cells plated on FITC-gelatin coated coverslips (pseudocolored *white*) for 2 h were treated with saracatinib as indicated (*left*) for 6 h. Cells were labeled to visualize F-actin (*red*), cortactin (*green*) and phosphotyrosine (*blue*). *Arrows* denote invadopodia and corresponding colocalized areas of focal matrix degradation with invadopodia components. Treatment with 1.0  $\mu\text{M}$  saracatinib resulted in F-actin aggregates lacking cortactin but accumulated at cytoplasmic sites where invadopodia typically occur (*arrowheads*). Bar, 10  $\mu\text{m}$ . **B.** UMSSC1 cells treated with increasing concentrations of saracatinib were stained as in **A** and quantified to determine the percentage of cells that produced functional invadopodia, presented as the mean  $\pm$  SEM. All treatment groups were significantly different from each another based on a one-way ANOVA ( $p < 0.05$ ) except 0 and 0.01  $\mu\text{M}$ , and 0.5 and 1.0  $\mu\text{M}$  pairs. **C.** Saracatinib decreases the ability of UMSSC1 cells to degrade ECM. The percentage of gelatin degradation per cell area for the cell population analyzed in B is shown with the mean  $\pm$  SEM.

**Figure 5** MMP9 secretion and ECM degradation activity in HNSCC cells is blocked by saracatinib. **A.** Localization of MMP9-containing vesicles in UMSSC1 invadopodia. *Top:* UMSSC1 cells plated on FITC-coated gelatin coverslips for 2 h were fixed and labeled with antibodies against cortactin and MMP9. The merged image indicates

areas of cortactin and MMP9 co-localization (*yellow; white arrows*) that correspond with sites of focal gelatin degradation (*black arrows*). Bar; 10  $\mu$ m, Asterisk; regions of global matrix degradation due to secreted protease activity. *Bottom*: Magnified view of indicated *Top* region. **B.** Inhibition of MMP9 secretion by saracatinib. Total cell lysates (*cell*) and aliquots of normalized conditioned media containing secreted MMPs (*sec*) from 1483 and UMSCC1 cells treated with increasing doses of saracatinib (*bottom*) were assayed for the presence of MMP2 and MMP9 by immunoblotting. Band intensities relative to control (0  $\mu$ M) are shown for each treatment condition; secreted MMP2 was not detected and therefore not quantified. **C.** Gelatin zymography of MMP9 activity. Representative zymograms from conditioned media of 1483 or UMSCC1 cells cultured with the indicated saracatinib concentrations (*bottom*). DMEM was used as a negative control (M), DMEM containing 10% FBS (FBS) was used as a positive control for zymography. *Graphs*, densitometric analysis of MMP9 zymography. Percentage of MMP9 gelatin clearing is represented and the mean  $\pm$  SEM for each cell line from three independent experiments.

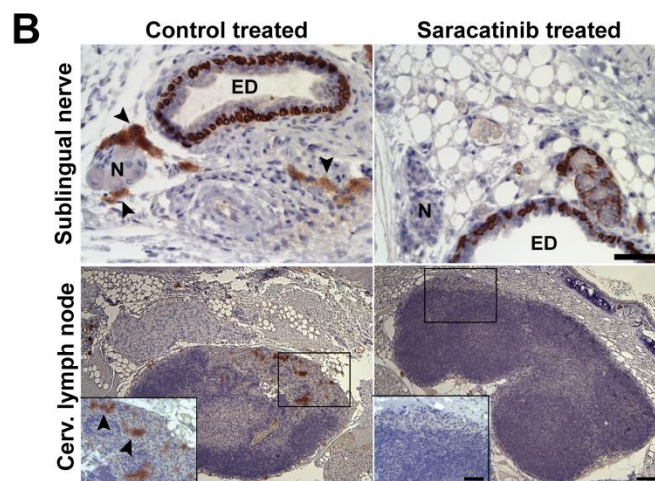
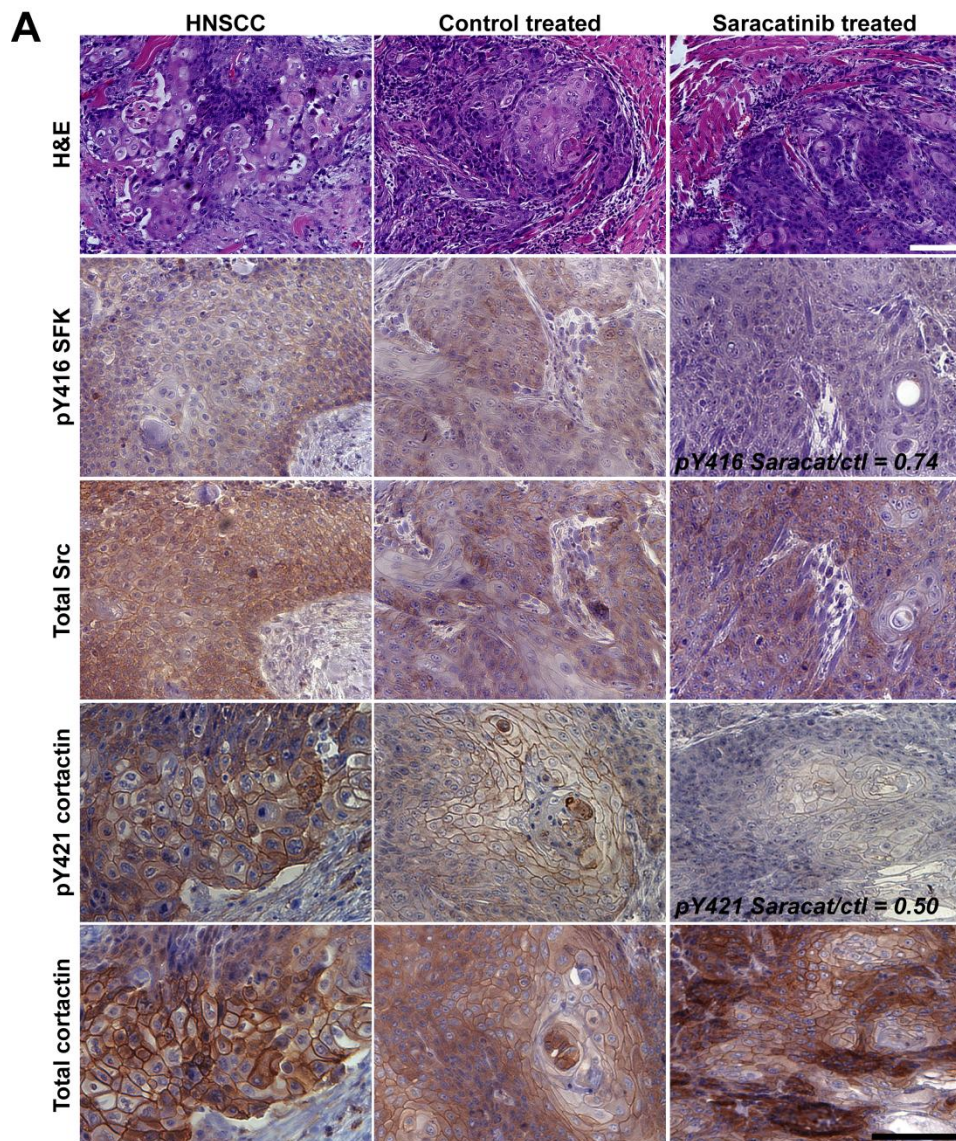


Ammer et al. Figure 1

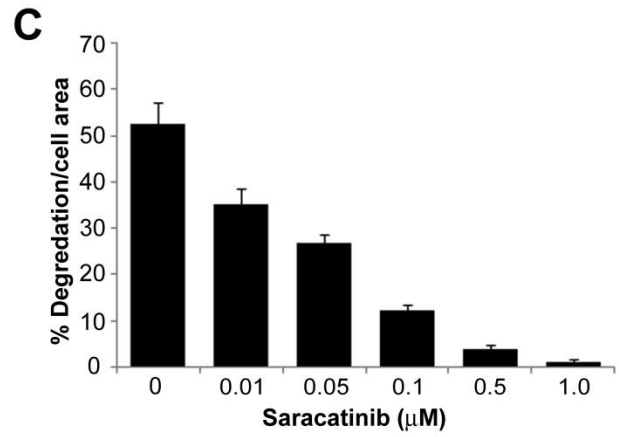
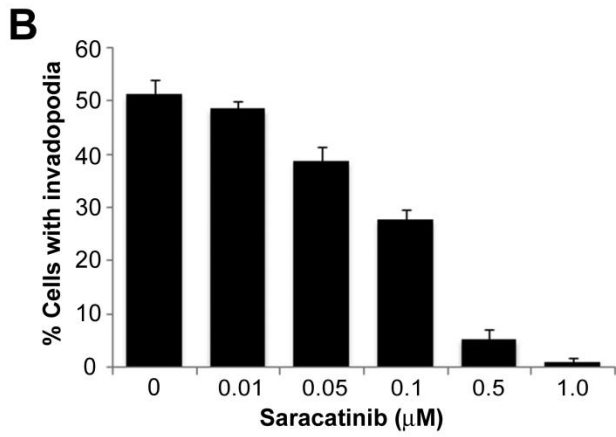
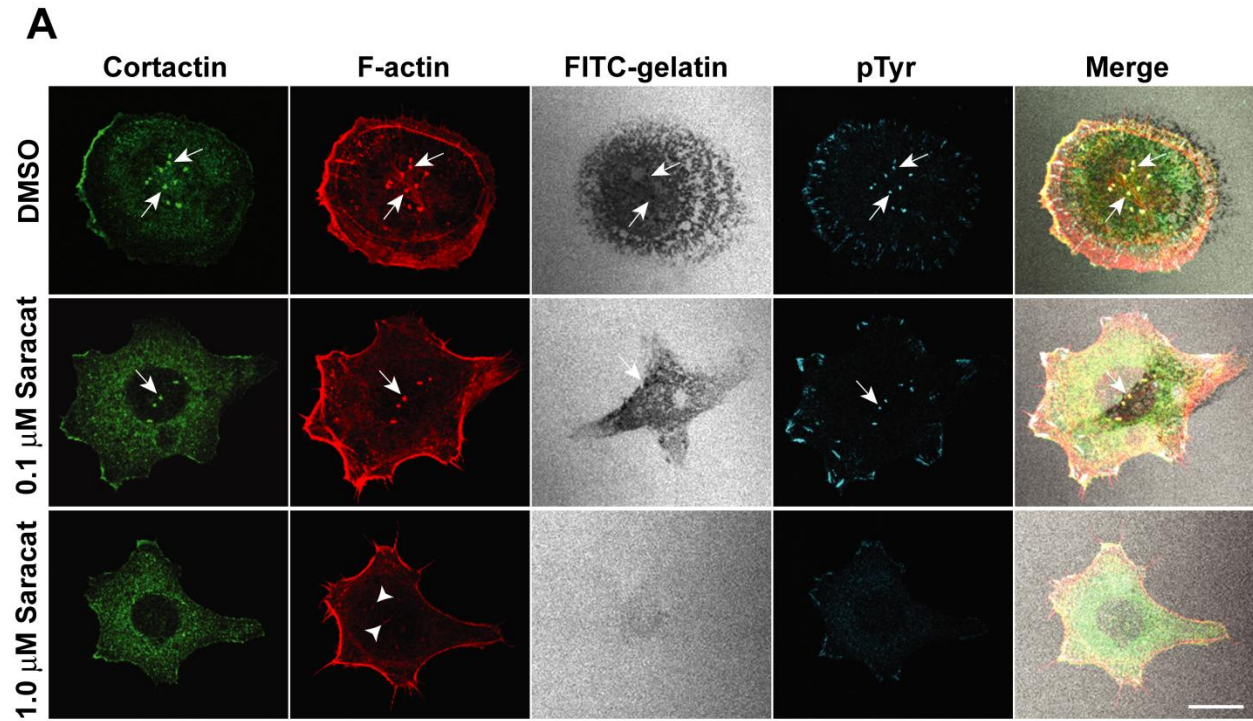


Ammer et al. Figure 2



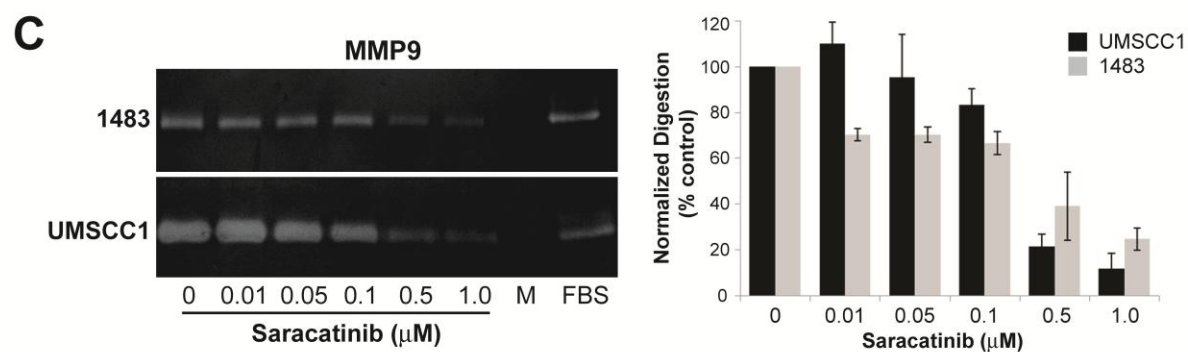
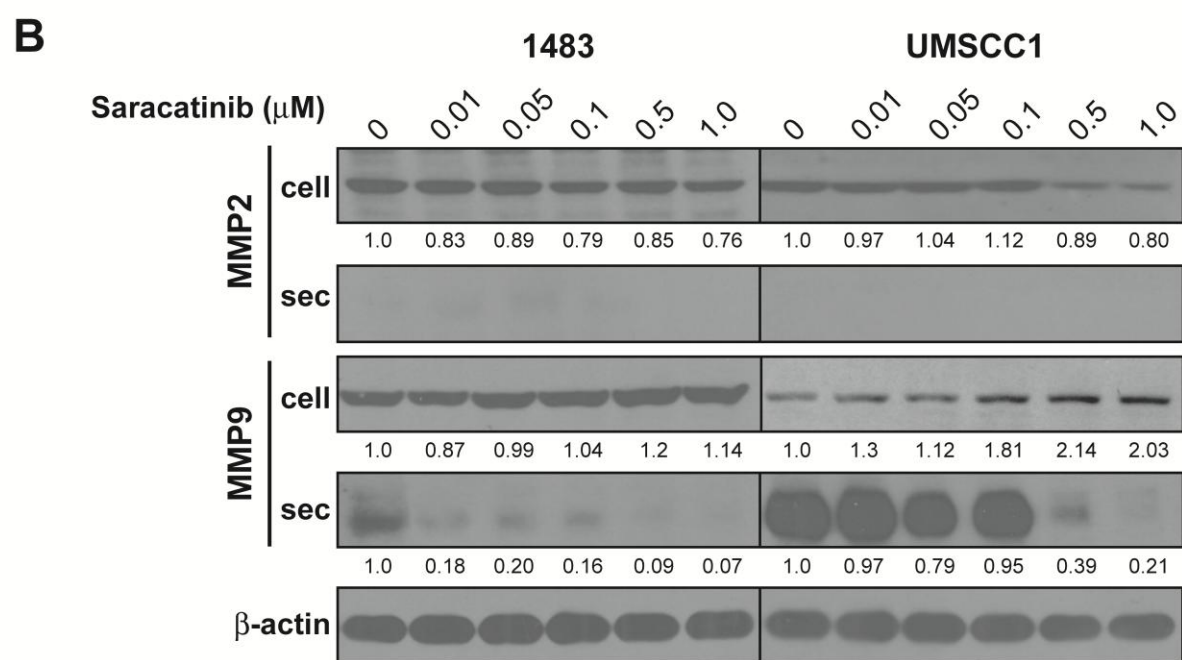
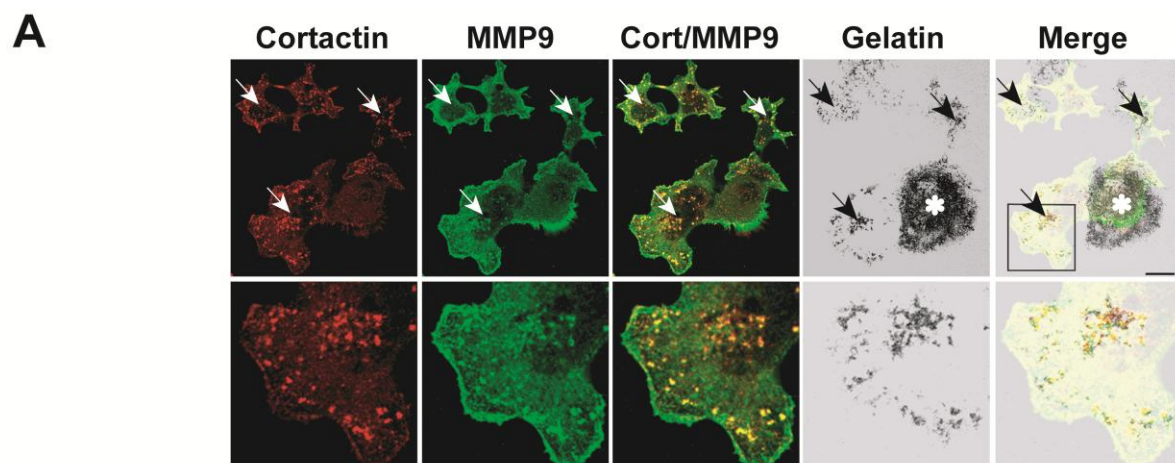


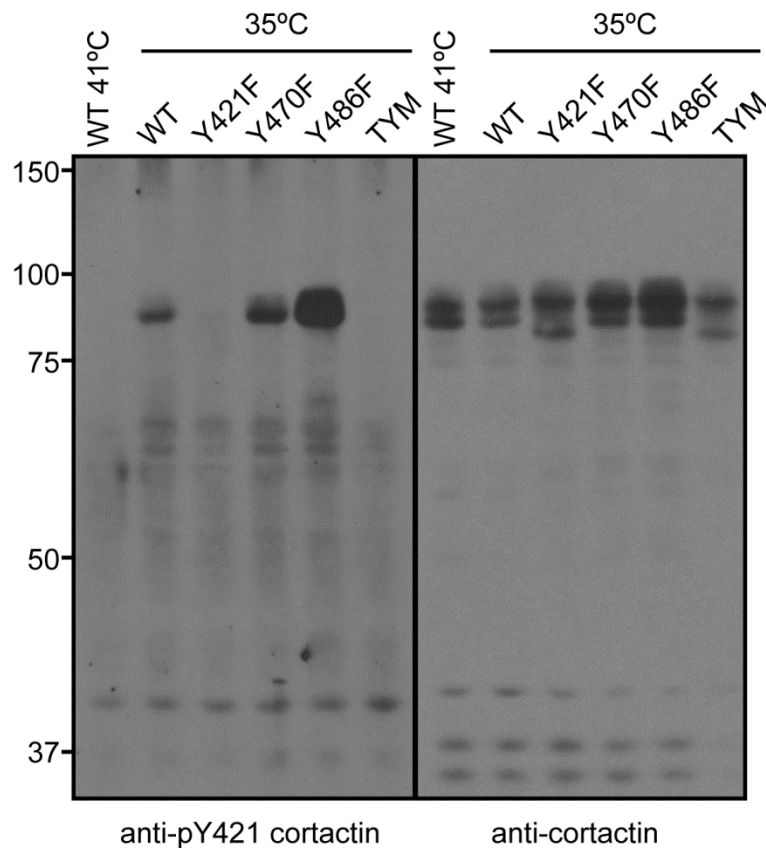
Ammer et al. Figure 3



*Ammer et al. Figure 4*







Ammer et al. Supplementary Figure 1

## **Supplementary Figure Legends**

**Supplementary Figure 1** Specificity of human anti-pY421 cortactin antibody. SYF fibroblasts lacking Src, Yes and Fyn were cotransfected with a the temperature-sensitive Src allele La29 along with expression vectors encoding recombinant wild-type human cortactin (WT) or cortactin mutants containing tyrosine-phenylalanine substitutions at the indicated Src-targeted codons and held at the non-permissive temperature (41°) or switched to the permissive temperature (35°). TYM; triple tyrosine-phenylalanine cortactin mutant lacking all three Src-targeted sites. Cells were lysed and analyzed by Western blot analysis with anti-pY421 cortactin (*left*). The blot was stripped and reprobed with anti-cortactin monoclonal antibody 4F11 (*right*). The position of molecular weight markers is noted on the *left* in kilodaltons.

# **Study 3: Cortactin Phosphorylated by ERK1/2 Localizes to Sites of Dynamic Actin Regulation and is Required for Carcinoma Lamellipodia Persistence**

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Running title: ERK1/2 regulates cortactin

## **Abstract**

### **Background**

Tumor cell motility and invasion is governed by dynamic regulation of the cortical actin cytoskeleton. The actin-binding protein cortactin is commonly upregulated in multiple cancer types and is associated with increased invasion and metastasis. Cortactin regulates actin nucleation through the actin related protein (Arp)2/3 complex, stabilizing the cortical actin cytoskeleton. Cortactin is regulated by multiple phosphorylation events, including phosphorylation of S405 and S418 by extracellular regulated kinase (ERK)1/2. ERK1/2 phosphorylation of cortactin has emerged as an important positive regulatory event, enabling cortactin to bind and activate the Arp2/3 regulator neuronal Wiskott-Aldrich syndrome protein (N-WASp), promoting actin polymerization and enhancing cell migration and tumor cell invasiveness.

### **Methodology/Principal Findings**

In this report we have developed phosphorylation-specific antibodies against cortactin phosphorylated at S405 and S418 to analyze the subcellular localization of this cortactin form in tumor cells and patient samples by microscopy. We evaluated the interplay between cortactin S405 and S418 phosphorylation with cortactin tyrosine phosphorylation in regulating cortactin conformational forms by Western blotting. Cortactin is simultaneously phosphorylated at S405/418 and Y421 in tumor cells, and through the use of point mutant constructs we determined that serine and tyrosine phosphorylation events lack any co-dependency. Expression of S405/418 phosphorylation-null constructs impaired carcinoma motility and adhesion, and also inhibited lamellipodia persistence monitored by live cell imaging.

### **Conclusions/Significance**

Cortactin phosphorylated at S405/418 is localized to sites of dynamic actin assembly in tumor cells. Concurrent phosphorylation of cortactin by ERK1/2 and tyrosine kinases enables cells with the ability to regulate actin dynamics through N-WASp and other effector proteins by synchronizing upstream regulatory pathways, confirming cortactin

as an important node in actin-based signal transduction. Reduced lamellipodia persistence in cells with S405/418A expression identifies an essential motility-based process reliant on ERK1/2 signaling, providing additional understanding as to how this vital signaling pathway impacts tumor cell migration.

## **Introduction**

Tumor cell motility and invasion is a central problem in cancer that is paramount in contributing to metastasis [1]. Tumor cells move through successive series of coordinated and integrated stages, with formation of protrusive membranous structures including filopodia, invadopodia and lamellipodia required for initiation and maintenance of invasion and migration [2,3,4,5]. Central to the movement of most carcinoma cell types undergoing single or collective migration is the production of lamellipodia at the leading edge of the cell. Lamellipodia are planar protrusive extensions of the plasma membrane produced by motile cells in two- and three-dimensional settings [6]. Lamellipodia extension drives cell migration through integrin-based adhesion with the underlying substratum, providing the necessary traction for contractile-based translocation of the cell body to generate productive movement [7]. It is generally accepted that dynamic regulation of the cortical actin cytoskeleton through cycles of actin polymerization and depolymerization are responsible for generating the propulsive force needed for lamellipodia extension [8].

The actin cytoskeleton within lamellipodia is governed by the activity of numerous actin-binding proteins. One element central to the formation of lamellipodia actin networks is activation of the actin-related (Arp) 2/3 complex, which nucleates filamentous (F-) actin polymerization within lamellipodia [9]. Arp2/3 complex binds to the sides of pre-existing F-actin, where upon activation the Arp2 and Arp3 subunits mimic the fast growing ("barbed" or "+") end of an actin filament, allowing for the rapid addition of actin monomers to the complex and subsequent filament extension [4]. The resulting Arp2/3-F-actin networks comprise an organized branched array of F-actin filaments that contribute to lamellipodia extension, with Arp2/3 localized at filament branch points [8,9]. Arp2/3 branch points are metastable, allowing for rapid breakdown of Arp2/3-F-actin networks by filament debranching [10]. Debranched F-actin filaments are further disassembled through the severing activity of members of the actin depolymerizing factor/cofilin family, which ultimately dissolve F-actin filaments to promote depolymerization, recycling actin monomers for additional rounds of polymerization and lamellipodia extension [11].



Arp2/3 activation is controlled by the activity of several actin nucleation promoting factors (NPFs). The best characterized NPFs to date are members of the Wiskott-Aldrich syndrome protein (WASp) family, which include the WASp and WAVE protein subgroups [12,13]. In many cell types, regulation of Arp2/3 activity by the WASp proteins N-WASp and WAVE2 are largely responsible for generating the actin network used for creating and regulating lamellipodia, filopodia and invadopodia [4,12,14], making these NPFs critical mediators of cell motility and invasion.

Another well-characterized NPF independent of the WASp protein family is the cortical actin-binding protein cortactin [15,16]. Cortactin directly binds Arp2/3 complex [17,18] and activates Arp2/3 complex nucleation activity in vitro, albeit at a lesser degree than WASp-family proteins [18,19]. In addition to actin nucleation, a unique function of cortactin is its ability to prevent spontaneous debranching of Arp2/3-F-actin networks by simultaneous binding to Arp2/3 and F-actin, prolonging the lifetime of branched filaments [19]. While the biochemical features of cortactin seem to point to a straightforward role in lamellipodia actin regulation, studies of cortactin function in lamellipodia have proven controversial, suggesting to a more complex role in cell migration. For instance, RNA interference studies have yielded conflicting results in regards to lamellipodia dynamics, with cortactin knockdown resulting in decreased lamellipodia stability and reduced persistence [20,21,22], whereas similar studies in different cell types suggest cortactin downregulation increases the length of extending lamellipodia [23]. Furthermore, recent analysis of fibroblast lamellipodia dynamics in cortactin-/- cells indicates that cortactin does not play a role in directly regulating lamellipodia protrusion or Arp2/3-based actin dynamics, but rather is important in mediating upstream activation of the small GTPases Rac1 and Cdc42, which in turn regulate WAVE2 and N-WASp activity [24]. While these reported discrepancies regarding cortactin function in lamellipodia have yet to be fully reconciled, it is clear that cortactin is an important regulator for normal and tumor cell migration in many cell systems [25,26]. An unambiguous role for cortactin has been shown in invadopodia, where removal of cortactin by RNA interference ablates invadopodia formation in multiple invasive tumor cell types [27,28,29].

Besides regulating Arp2/3-based cortical actin networks by direct interactions, cortactin also functions as a key mediator in several kinase-based signal transduction cascades that serve to indirectly govern Arp2/3 activity and subsequent cell movement. Cortactin is a well-defined target for Src kinase [30], phosphorylating human cortactin on tyrosine residues Y421, Y470 and Y486 within the proline-rich (PR) carboxyl-terminal domain [31]. Several other tyrosine kinases target these residues [32,33,34,35], indicating that they collectively form a “hot spot” region as a point of convergence for multiple signaling pathways. Cortactin phosphorylated at tyrosines 421, 470 and/or 486 creates Src homology (SH)2 docking sites for several phosphorylating kinases, as well as the adaptor proteins Crk [36] and Nck1 [37]. In the case of Nck1, Nck1/cortactin complexes interact with N-WASp or WASp interacting protein through the Nck1 SH3 domain to stimulate Arp2/3-dependent actin nucleation [37], which in cooperation with Arg kinase has been recently demonstrated to be important for regulating lamellipodia protrusion and leading edge adhesion formation [38]. The cortactin/Nck1 complex is also required to stimulate actin polymerization essential for invadopodia function in invasive breast cancer cells [39]. These studies are in agreement with the localization of tyrosine phosphorylated cortactin within lamellipodia and invadopodia [40], lending mechanistic insight into the long recognized pro-migratory and pro-invasive properties associated with cortactin tyrosine phosphorylation [29,31,41,42].

In addition to tyrosine phosphorylation, cortactin is a target for multiple serine/threonine kinases [43]. Stimulation of tumor cells with epidermal growth factor (EGF) results in increased serine/threonine phosphorylation of serine residues 405 and 418 within the PR domain, coincident with a characteristic shift in cortactin electrophoretic mobility from 80 kDa to 85 kDa in SDS-PAGE [44,45]. The mobility shift and phosphorylation of S405/S418 are impaired by pharmacologic inhibition of mitogen activated protein/extracellular signal regulated kinase kinase (MEK)1/2, and direct biochemical evidence indicates that the MEK effector kinases ERK1/2 directly phosphorylate cortactin at these sites [45]. Phosphorylation of S405/S418 enhances binding of the cortactin carboxyl-terminal SH3 domain to N-WASp and activates N-WASp NPF activity, indicating a functional role in stimulating Arp2/3-mediated actin dynamics independent of tyrosine phosphorylation [46]. This is supported by studies expressing

phosphorylation-null and phosphomimetic point mutant constructs in cells, suggesting that S405/S418 phosphorylation plays a critical role in regulating cellular actin polymerization necessary to promote cell motility [47] and invadopodia function [29]. In addition, p21 activated kinase 1 (PAK1) phosphorylates cortactin at S405/S418, serving to stimulate N-WASp activity required for clathrin-independent endocytosis [48]. While studies to date implicate a positive regulatory function for cortactin S405/418 phosphorylation in promoting N-WASp-mediated Arp2/3 actin structures, the subcellular localization of phosphorylated S405/418 cortactin, as well as the precise role S405/418 phosphorylation plays in regulating lamellipodia dynamics have not been evaluated.

In this study, we have generated site-specific antibodies against phosphorylated cortactin S405 and S418 to determine the spatial and temporal localization of cortactin in dynamic actin structures and human tumors, and to evaluate signaling interplay between cortactin tyrosine and serine phosphorylation events. We also determined the effects of S405/418 cortactin phosphorylation on EGF-induced cell migration, adhesion and lamellipodia dynamics in carcinoma cells.

## **Materials and Methods**

### **DNA Constructs and siRNA**

For Myc-tagged human cortactin expression constructs, the wild-type human cortactin cDNA subcloned into pcDNA FLAG2AB [49] was used as a template for producing point mutants by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA). Codon alterations in human cortactin were: S405A, S418A, S405A/S418A, Y421F, Y470F, Y486F, Y421F/Y470F/Y486F and W492K. Cortactin cDNAs were amplified by PCR as BamHI-EcoRI fragments and subcloned into BamHI-EcoRI digested pRK5Myc [50]. Murine GFP-tagged expression constructs were produced using pcDNA3FLAG2AB wild-type murine cortactin [17] as the template for mutagenesis, then subcloned as EcoRI-KpnI PCR fragments into pAcGFP-C1 (Clontech, Mountain View, CA). The temperature-sensitive vSrc LA29 construct was previously described [51]. mCherry- $\beta$ -actin was obtained from D. Schafer (University of Virginia), with the parent construct produced by R. Tsien (University of California, San Diego). Small interfering (si)RNA targeting rodent cortactin (5'-GCTTCGAGAGAATGTCTTC-3') was purchased from Thermo Scientific (Waltham, MA)

### **Cell lines and Transfection**

The HNSCC cell lines 1483 [52], UMSCC1 and UMSCC2 [53] were maintained as described [41]. SYF cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the supplied protocol. The rat mammary adenocarcinoma line MTLn3 was maintained in aMEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin. Transient transfections were conducted with  $3 \times 10^6$  cells and 2 $\mu$ g of plasmid construct or siRNA using the Nucleofector I device (Amaza Biosystems, Berlin, Germany).

### **Antibodies**

Antibodies against phosphorylated serine 405 (pS405) and serine 418 (pS418) of human cortactin were produced by 21<sup>st</sup> Century Biochemicals (Marlboro, MA). Synthetic phosphorylated cortactin peptides containing the sequences NH<sub>2</sub>-

KTQTPPV[pS]PAPQPTC-COOH (cortactin pS405) and NH<sub>2</sub>-TEERLPS[pS]PV-COOH (cortactin pS418) were produced, conjugated to keyhole limpet cyanine and injected into rabbits. Immune serum was screened by enzyme-linked immunosorbent assay against the appropriate phosphorylated cortactin peptide coupled to bovine serum albumin. High-titer bleeds were identified for each peptide, and immune serum was passed two successive times through chromatography columns containing agarose beads coupled to the equivalent non-phosphorylated peptide. The flow through material for each peptide was subsequently passed twice through chromatography columns containing beads conjugated to the matched phosphorylated cortactin peptide. After extensive washing, bound antibodies for each phosphorylation site were eluted, concentrated and screened for specificity by Western blotting against recombinant cortactin mutant proteins harboring alanine-serine point mutations at serine 405 or 418, respectively (Fig. 1A). The anti-pS405 and anti-pS418 cortactin antibodies are currently available through Protea Biosciences (Morgantown, WV). Anti-cortactin (4F11) was used as described [41]. Anti-pY421 cortactin and anti-pY418 Src were from Invitrogen (Carlsbad, CA). Anti-ERK1/2 and pERK1/2 were from Cell Signaling (Danvers, MA). Anti-Myc epitope tag (4A6) was from Millipore (Billerica, MA). Anti-GFP (JL-8) was from Clontech (Mountain View, CA) and anti- $\beta$ -actin was from EMD4Biosciences (San Diego, CA).

### **Western blotting and Immunoprecipitation**

Western blotting was conducted as described [41]. Primary antibody dilutions used were: anti-pS405 cortactin (1:4000), anti-pS418 cortactin (1:500), anti-cortactin (1:1000), anti-pY-421 cortactin (1:2000), anti-ERK1/2 (1:2000), anti-pERK (1:2000), anti-pY418 Src, anti-GFP (1:1000) and anti- $\beta$ -actin (1:5000). Immunoprecipitations were performed as described [40] using 5 $\mu$ g of precipitating antibody captured with 40 $\mu$ l of a 50% Protein A/G bead slurry (Thermo Fisher Scientific, Pittsburgh, PA). In some cases cells were treated with selumetinib (AZD6244; ARRY-142886) or saracatinib (AZD0530) for 24h prior to immunoprecipitation and Western blotting analysis.

## Microscopy

UMSCC2 cells were plated on fibronectin-coated coverslips (10µg/ml; Sigma, St Louis, MO) and allowed to attach before serum starvation for 16 h. Cells were stimulated with 100ng/ml EGF (Millipore) for 1h before fixation. UMSCC1 cells plated on FITC-gelatin (Sigma) for 8 h were processed for confocal microscopy using Zeiss LSM 510 Meta system (Thornwood, NY) as described [49]. Anti-pS418 cortactin was used at 1:1000, 4F11 at 1:500 and rhodamine-conjugated phalloidin at 1:1000 (Invitrogen, Carlsbad, CA).

For immunohistochemistry, HNSCC tissue blocks were obtained from the West Virginia University Tissue Bank and used under approval of the West Virginia University Institutional Review Board. Five-micrometer sections from formalin-fixed, paraffin-embedded blocks were processed for immunostaining using the Discovery XT automated staining system (Ventana, Tucson AZ). Briefly, after deparaffinization and antigen retrieval, sections were incubated with monoclonal rabbit anti-cortactin (Novus, Littleton, CO) at 1:2000, anti-pS418 cortactin at 1:25 and anti-pERK1/2 at 1:100 dilutions. All primary antibodies were incubated in Dako diluent (Dako, Carpinteria, CA) for 1 h. Primary antibodies were detected with the Omnimap antibody horseradish peroxidase kit (Ventana). Slides were counterstained with hematoxylin and post-counterstained with bluing reagent (Ventana). Images were visualized with an Olympus AX70 microscope and captured using the MicroBrightfield system (Williston, VT).

Live cell imaging was conducted using MTLn3 cells starved for 3 h with serum-free media prior to stimulation with 100ng/ml EGF. Cells were plated on delta-T glass bottom dishes (Fisher) coated with 10µg/ml fibronectin (Sigma). Immediately following EGF addition, cells were imaged by differential interference contrast using a Nikon TE2000 inverted microscope equipped with a Roper CoolSNAP HQ charge-coupled device camera (Photometrics, Tucson, AZ). Images were captured every 5 s for 15 min (181 total frames). A Nikon LiveScan SFC swept field microscope was used for imaging cells expressing mCherry-actin. In all cases, GFP-cortactin expressing cells were identified by fluorescence microscopy prior to imaging. Kymograms were produced by

extracting 1 pixel-width strips from each movie frame at points of initial and maximal lamellipodia extension, and assembled using ImageJ (v1.40).

### **Electric Cell Substrate Impedance Sensing**

To assay cell motility and adhesion,  $5 \times 10^5$  cells were plated into 8-well electric cell substrate impedance sensing dishes (ECIS; Applied Biophysics, Troy, NY). For motility measurements, cells were allowed to adhere overnight on 8W1E dishes to form a monolayer. Adhesion was assayed immediately after plating cells onto 8W10E dishes. Measurements were conducted for 24 h at 45kHz, with reading taken at 1 min intervals. Cells treated with selumetinib were serum starved 24 h in the presence of drug prior to ECIS.

### **Statistical Analysis**

Differences in mean groups for migration, adhesion and kymography between control and treated groups were evaluated using one way ANOVA, followed by Student-Newman-Keuls post hoc testing. All differences were considered significant at  $p < 0.05$ . A minimum of three experimental groups were used for all analyses.

## **Results**

### **Localization of pS418 cortactin with dynamic cortical actin structures**

We developed antibodies specific to phosphoserine 405 (pS405) and phosphoserine 418 (pS418) of human cortactin to facilitate analysis of these sites. To validate antibody specificity, epitope (Myc)-tagged cortactin constructs containing wild-type (WT) cortactin, cortactin with individual serine to alanine mutations at serine 405 (S405A), 418 (S418A) or with both mutated in tandem (S405,418A) were produced and transfected into 1483 cells. Total cell lysates were blotted with anti-pS405 or anti-pS418 antibodies (Figure 1A). The anti-pS405 antibody recognized the WT and S418A cortactin variants, failing to blot constructs containing the S405A mutation. Conversely, anti-pS418 blotted WT and S405A, failing to recognize cortactin constructs with S418A mutations. All cortactin variants were recognized by an anti-cortactin monoclonal antibody (Figure 1A), indicating equivalent expression of the assayed constructs. These results indicate that the anti-pS405 and anti-pS418 antibodies specifically recognize their cognate phosphorylated cortactin epitope, and that no interdependence exists between phosphorylation of cortactin S405 and S418.

To determine the subcellular localization of serine phosphorylated cortactin, we conducted indirect immunofluorescence studies on cells producing lamellipodia and invadopodia, two actin-based structures that depend in part on N-WASp activity. While the anti-pS405 antibody yielded non-specific staining in our hands (data not shown), anti-pS418 specifically labeled lamellipodia and cytoplasmic puncta (presumably vesicles) in UMSCC2 cells. In cells with a motile phenotype, anti-pS418 localized with cortactin and F-actin in these regions (Figure 1B, top row). Labeling of UMSCC1 cells plated on FITC-coated gelatin matrix with anti-pS418 indicated specific localization to a subset of invadopodia that coincided with cortactin, F-actin and areas of gelatin clearing indicative of matrix metalloproteinase mediated invadopodia activity (Figure 1B, middle and bottom rows).



In solid human tumors, cortactin and cortactin phosphorylated on tyrosine 421 (pY421) localizes to invasive tumor fronts and to cell-cell junctions [41,49]. To determine the location of pS418 cortactin in tumor tissue, head and neck squamous cell carcinoma (HNSCC) cases were sectioned and stained with anti-pS418 (Figure 1C). Cortactin pS418 was abundant in HNSCC cell cytoplasm and was enriched in areas of cell-cell contact, displaying a pattern similar to sections labeled with a total cortactin antibody (Cort). These tumor regions also contained activated ERK1/2, as evidenced by pronounced cytoplasmic and nuclear staining of phosphorylated ERK1/2 in serial sections (Figure 1C).

### **Growth factor mediated phosphorylation of cortactin S405/418 is MEK dependent**

Previous biochemical work has implicated chemical inhibition of MEK and subsequent blocking of ERK1/2 activation as a major pathway responsible for cortactin S405/418 phosphorylation [45]. To further evaluate the role of the MEK-ERK1/2 pathway on cortactin phosphorylation, we utilized the anti-pS405 and pS418 cortactin antibodies to directly test the effects of MEK inhibition on cortactin pS405/418. Western blot analysis of cell extracts from EGF- and serum-stimulated UMSCC1 cells with anti-pS405 and pS418 antibodies displayed similar phosphorylation kinetics of S405 and S418, with phosphorylation of both sites first evident 10 min after stimulation (Figure 2A) and remaining phosphorylated up to 2 h (data not shown). Treatment of UMSCC1 or 1483 cells with the small molecule MEK inhibitor selumetinib [54] reduced EGF-stimulated cortactin S405/418 phosphorylation in a dose-dependent manner, where near elimination of phosphorylation at both serine residues occurred at doses  $\geq 1\mu\text{M}$  (Figure 2B). ERK1/2 activity was also reduced under similar dose conditions, although complete ablation of ERK1/2 phosphorylation was observed at doses  $\geq 5\mu\text{M}$  (Figure 2B). These data suggest that the MEK-ERK pathway is largely responsible for growth-factor induced cortactin S405/418 phosphorylation in HNSCC cells, in agreement with previous findings in other cell types [45].

## **The 80kDa to 85kDa cortactin conformational shift is associated with serine and tyrosine phosphorylation**

Based on sequence analysis, the largest and most prominent cortactin isoform (cortactin “A” or “SV1”) encodes a 61.5kDa protein [55,56]. This cortactin form frequently migrates as an 80/85kDa doublet in SDS-PAGE [30,57] that has been attributed to conformational alterations within the polypeptide chain [15,45]. Shifting from the 80kDa to 85kDa form is seen in response to EGF, with the resulting 85kDa band associated with S405/418 phosphorylation [44,45]. To directly assess the presence of pS405/418 in the two cortactin conformational isomers, serum-starved UMSCC2 (Figure 3A) and 1483 (Figure 3B) cells were stimulated with EGF and the cortactin forms in cell lysates were analyzed at successive time points with anti-pS405 and anti-pS418 antibodies. S405/418 phosphorylation was maintained in the 85kDa cortactin form in both cell lines following serum starvation, despite of the lack of ERK1/2 activity (0 min, Figure 3A and Figure 3B). EGF stimulation resulted in complete conversion of the 80kDa to the 85kDa cortactin form by 1 h after EGF treatment in both cell lines (Figure 3A and Figure 3B). Cortactin pS405 and pS418 was observed primarily in the 85kDa form and increased at both sites during the entire time course, whereas ERK1/2 activity peaked at 15 min and rapidly declined afterwards (Figure 3A and Figure 3B). Interestingly, the phosphorylation of S405 was also associated with an increase appearance of cortactin degradation in UMSCC2 cells (Figure 3A). It is uncertain whether these products represent increased overall cortactin degradation, or if the net cortactin degradation is constant but is selectively identified by the pS405 antibody in response to EGF treatment and phosphorylation. EGF-induced Src activation and cortactin pY421 phosphorylation was sustained throughout the entire time course in UMSCC2 cells (Figure 3A), indicating that cortactin can be simultaneously phosphorylated by ERK1/2 and Src or potentially other EGF-stimulated cortactin tyrosine kinases. Pretreatment of UMSCC2 cells with the Src family kinase inhibitor saracatinib at 10 $\mu$ M or selumetinib at 1 $\mu$ M completely impaired the cortactin shift from 80kDa to 85kDa (Figure 3C). The exclusive presence of pS405 and pS418 in the EGF-induced 85kDa cortactin form, as well as the ability of MEK inhibition to impair the cortactin shift is consistent with results obtained from previous work [45]. Our

results also identified EGF-induced Src-mediated phosphorylation of cortactin at tyrosine 421 as a necessary mediator of the cortactin shift.

### **Cortactin serine phosphorylation *in vivo* is independent from tyrosine phosphorylation**

EGF treatment of UMSCC2 cells resulted in phosphorylation of cortactin S405/418 and cortactin pY421 (Figure 3A). A previous *in vitro* study evaluating the impact of cortactin phosphorylation on N-WASp activation determined that S405/418 phosphorylation by ERK1/2 enables the cortactin SH3 domain to stimulate N-WASp Arp2/3 activation, while Src phosphorylation downregulates N-WASp activity and counteracts the effects of S405/418 phosphorylation [46]. This proposed “on-off switch” postulates that cortactin serine and tyrosine phosphorylation are mutually exclusive events governing the ability of cortactin to regulate N-WASp activity and downstream actin reorganization [58]. Using the available antibodies reactive against cortactin pS405 and pY421, we sought to determine if these two different classes of phosphorylation events are interdependent in any manner. Cortactin depleted SYF fibroblasts (null for the Src, Yes and Fyn kinases) were co-transfected with the temperature-sensitive vSrc construct *tsLa29*-GFP [51] to activate the Src and ERK1/2 signaling pathways, along with constructs encoding wild-type cortactin or the following Myc-tagged cortactin mutants: Y421F, Y470F, Y486F, Y421/Y470/Y486F (TPM), S405A, S418A, S405/418A (Figure 4A). A W492K cortactin mutant was also included, as this mutant abolishes the ability of the cortactin SH3 domain to interact with corresponding SH3 binding proteins [59]. After shifting to 35°C for 2 h to activate *tsLa29*-GFP, the serine and tyrosine cortactin mutants were analyzed for phosphorylation at Y421 and S405 by SDS-PAGE and Western blotting (Figure 4B). Mutations to S405 and S418 alone and in combination did not impact the ability of these constructs to be phosphorylated on Y421, as indicated by their recognition with the anti-pY421 antibody (Figure 4B). Similarly, mutations to Y421, Y470, and Y486, alone and in combination (TYM) did not affect the ability of these constructs to be phosphorylated on S405. These data indicate that cortactin is simultaneously phosphorylated at S405 and Y421 downstream of vSrc activation, suggesting in this system that phospho-regulation of cortactin SH3 domain function is

not governed *in vivo* by the serine-tyrosine “on-off switch” mechanism proposed from previous *in vitro* experimentation [46,58].

### **S405/418 phosphorylation is required for efficient tumor cell motility and adhesion**

To evaluate the role of cortactin S405/418 phosphorylation on carcinoma cell migration, 1483 and UMSCC1 cells were treated with selumetinib and assayed for effects on motility by ECIS (Figure 5). Selumetinib treatment impaired the motility of both cell types in a dose-dependent manner, corresponding to the observed decreases in S405/418 phosphorylation (Figure 2B). Since MEK inhibition likely impaired the phosphorylation of other proteins involved in motility in addition to cortactin, we directly assessed the impact of cortactin S405/418 phosphorylation on cell migration using phosphorylation-null cortactin expression constructs. MTLn3 rat mammary adenocarcinoma cells were initially transfected with a siRNA targeted against rodent cortactin, followed by transfection with GFP-tagged human wild-type (WT), S405A, S418A and S405/418A cortactin constructs. Cortactin siRNA reduced endogenous cortactin levels to > 90%, having no impact on expression of the human GFP-labeled variants (Figure 6A). MTLn3 cells with cortactin knockdown (si) displayed a 29% reduction in motility compared to control (Ctl) (Figure 6B). Expression of wild-type human GFP-cortactin (WT) led to a 2-fold increase in motility, presumably due to increased expression of this variant over endogenous (Ctl) levels (Figure 6A). Expression of S405A, S418A or S405/418A cortactin resulted in an 49% average decrease in cell migration for each cortactin mutant, indicating that phosphorylation of S405 and S418 are both vital in maintaining optimal carcinoma cell motility (Figure 6B). Since lamellipodia formation is required for detached cells to adhere to the ECM, we conducted ECIS assays to determine the effects of cortactin S405/418 phosphorylation on cell adhesion. MTLn3 cells lacking cortactin expression (si) exhibited a 50% decrease in cell adhesion compared to control (Ctl) cells. Expression of wild type (WT) GFP-cortactin restored adhesion to levels similar to Ctl, whereas expression of S405A, S418A or S405/418A cortactin mutants all reduced adhesion to levels 42-58% of Ctl, failing to restore adhesion to levels above cortactin si cells (Figure 6C). These results

suggest that S405/418 phosphorylation is critical for carcinoma cell motility and adhesion, representing an important pro-migratory substrate targeted by the MEK-ERK1/2 pathway.

### **Cortactin S405/418 phosphorylation is required for carcinoma cell lamellipodia persistence**

Given the localization of pS418 cortactin within lamellipodia (Figure 1B) and the effects of cortactin S405/418A expression on cell motility, we evaluated the impact of cortactin S405/418 phosphorylation on lamellipodia dynamics using live-cell imaging and kymographic analysis. Serum-starved MTLn3 cells expressing mCherry- $\beta$ -actin and containing endogenous cortactin knockdown alone (si), rescued with human GFP- wild-type cortactin (si+WT) or with GFP-cortactin S405/418A (si+S405,418) were stimulated with EGF for 15 min. Lamellipodia dynamics were monitored by time lapse video microscopy (Figures S1-S4) and assayed by kymography (Figure 7A). EGF-stimulated MTLn3 cells produced an initial dominant lamellipodia that reached maximal extension between 1.5 and 3 min, and retracted to the point of origin between 5-7 min [60,61]. Control MTLn3 cells containing mCherry- $\beta$ -actin displayed similar extension-retraction kinetics when assayed by kymography (Figure 7B and Figure S1). While no differences were observed in lamellipodia protrusion rates in any of the assayed cellular conditions (Figure 7A), cortactin knockdown (si) increased lamellipodia extension by an average of 5.8  $\mu$ m over the maximum extension length observed in control cells (Figure 7A and B). Lamellipodia formed in cortactin si cells failed to effectively retract lamellipodia, demonstrating a ~2-fold increase in average lamellipodia persistence over control levels (Figure 7A and Figure S2). These results are consistent with the observed increase in lamellipodia extension and persistence observed when MTLn3 cells contact EGF-coated bead matrices [23]. These effects are fully rescued to control levels upon expression of WT GFP-cortactin (si+WT; Figure 7A and B). Although expression of GFP-cortactin S405/418A in cortactin si cells did not impact EGF-induced lamellipodia extension, average lamellipodia persistence was reduced by 46%, from 195 sec in si+WT cells to 106 sec in si+405,418 cells (Figure 7A). The lamellipodia in si+405,418 cells displayed series of multiple short extensions and retractions, had enhanced ruffling

and appeared more labile than control or si+WT cells (Figure 7B: Figures S1 and S2 compared to Figure S4). These results suggest that S405/418 phosphorylation is vital in regulating lamellipodia actin dynamics responsible for proper protrusive behavior.

## **Discussion**

While the effects of cortactin phosphorylation at S405 and S418 by ERK1/2 have been studied at the biochemical and functional level in several systems [29,45,46,47], the spatial and temporal evaluation of S405 and S418 phosphorylation have been hampered due to the lack of suitable reagents to directly study these sites in cellular and tissue contexts. Our development of anti-pS405 and anti-p418 cortactin antibodies has allowed us to examine the localization and signaling pathways regulating these cortactin phosphorylation events. These antibodies, coupled with the use of phosphorylation-null mutant constructs, allowed us to validate and extend previous findings implicating these sites in the regulation of carcinoma cell motility and associated lamellipodia dynamics.

The localization of pS418 cortactin in carcinoma lamellipodia and invadopodia is consistent with the defined and emerging roles cortactin plays in regulating actin dynamics within these structures [26,62]. To date, all studies designed to evaluate the cellular effects of pS405/418 phosphorylation have relied on the use of phosphorylation null or phosphomimetic (S405/418D) constructs. In pancreatic tumor cells, S405/418A and S405/418D both promote lamellipodia protrusion over control levels, whereas S405/418A inhibits and S405/418D promotes cell motility [47]. While the ability of S405/418A to promote lamellipodia protrusion in these studies is unclear, the remaining results are consistent with an activating role for S405/418 phosphorylation in lamellipodia dynamics and motility. Similar results were obtained in the analysis of S405/418 on invadopodia function, with S405/418A expression impairing and S405/418D promoting ECM degradation activity [29]. Phosphorylation of cortactin S418 within lamellipodia and invadopodia (Figure 1B) supports these results. Precisely where cortactin is phosphorylated on S405/418 in carcinoma cells remains to be determined, although the phosphorylating kinases ERK1/2 and PAK1 have been localized within lamellipodia [63,64] and invadopodia [65]. This could suggest that cortactin is initially localized to lamellipodia or invadopodia, where it is subsequently phosphorylated on S405/418 when associated with the cortical actin networks within these structures. An

analogous mechanism is employed for cortactin tyrosine phosphorylation within lamellipodia [40].

In HNSCC and several other tumor types, cortactin is present in the cytoplasm and is enriched at cell-cell junctions [41,66,67]. The localization of pS418 cortactin at regions of HNSCC cellular contact within tumors resembles the localization pattern of pY421 cortactin in this tumor type [68]. The staining pattern of cortactin and its tyrosine phosphorylated form is reminiscent of that found in two-dimensional epithelial monolayers, where cortactin has been shown to be essential for Arp2/3-mediated actin remodeling resultant from E-cadherin homoligation and subsequent Src activity [69,70]. While the presence of pS418 cortactin at these sites suggests additional functional roles for cortactin in E-cadherin-mediated actin regulation within tumors, whether or not cortactin S405/418 phosphorylation impacts elements of E-cadherin-based regulation of solid tumor behavior (such as tumor cell cohesion, motility or dissemination) remains to be examined.

Selumetinib inhibition of cortactin S405/S418 phosphorylation is consistent with results obtained with non-clinical MEK inhibitors [29,45], reinforcing the MEK-ERK1/2 pathway as the main signaling route responsible for phosphorylating these cortactin sites in tumor cells. This is supported by direct phosphorylation of cortactin by ERK1/2 in vitro [45] along with our data demonstrating concomitant downregulation of active ERK1/2 resultant of selumetinib treatment. In addition to MEK, PAK1 has recently been shown to phosphorylate cortactin at S405/418, regulating N-WASp actin dynamics responsible for clathrin- and caveolin-independent endocytosis [48]. PAK1 is activated primarily by binding to active Cdc42 or Rac1 [71], although alternative modes of activation have also been described [72]. Activated PAK1 also binds and activates MEK, stimulating ERK1/2 activation [73]. Since MEK inhibition largely ablates S405/418 phosphorylation in most cell types, the impact of PAK1 activity on S405/418 phosphorylation may be context dependent, with direct PAK1 phosphorylation of cortactin S405/418 regulating actin polymerization required for endosomal trafficking, while MEK-mediated phosphorylation (activated by Raf or other MEK activators) may be primarily responsible for governing motility-based actin dynamics. In addition, the related kinase PAK3



phosphorylates cortactin at S113, an event that downregulates the ability of cortactin to bind F-actin and is important in modulating invadopodia function [29,74]. While our understanding regarding the interrelationship and regulation between PAK and MEK in governing cortactin S405/418 phosphorylation is currently incomplete, it is clear that the PAK- MEK-ERK1/2 signaling nexus impinges at multiple levels on cortactin to regulate actin dynamics involved in several membrane-based cellular processes.

Consistent with other reports [44,45], we observed the MEK-dependent EGF-induced shifting of cortactin from the 80kDa to 85kDa form by Western blotting. Direct analysis with anti-pS405 and anti-pS418 antibodies indicates that the 85kDa form is almost exclusively phosphorylated on these residues, as was determined by <sup>32</sup>P labeling and tryptic peptide analysis [44,45]. The shift in cortactin  $M_r$  is not attributable to bulk addition of phosphate, since phosphatase treatment of cortactin immunoprecipitates from EGF-treated cells failed to reconvert the 85kDa form to 80kDa (data not shown). While the distinct 80kDa and 85kDa bands represent different post-translationally modified cortactin forms associated with pS405/418 phosphorylation, mutations at these sites have no effect on 80/85kDa cortactin ratios, with the S405/418A mutant displaying a similar cortactin electrophoretic pattern to wild type cortactin (Figure 4). This suggests that S405/418 cortactin phosphorylation, while associated with the shift from 80 to 85kDa, is not *necessary* for generation of the 85kDa cortactin form. This is supported by the presence of 80kDa and 85kDa cortactin forms produced in kinase-free systems [30,75] and by the existence of a single 85kDa form when analyzed by urea denaturing SDS-PAGE [75].

The lack of detailed structural data for cortactin derived by nuclear magnetic resonance spectroscopy or X-ray crystallography has hindered the field in understanding conformational changes cortactin undertakes in response to post-translational modifying events. The existence of cortactin in a “closed” versus “open” form regulated by S405/418 phosphorylation has been proposed to explain the observed 80 to 85kDa shift [45]. Support for this is derived from biochemical studies on N-WASp activation by the cortactin SH3 domain, where S405/418 phosphorylation enhances N-WASp activation and Arp2/3 actin nucleation activity [46]. These studies propose that the “closed”

cortactin form undergoes an autoinhibitory conformational state where the carboxyl terminal helical proline-rich (HP) domain containing S405 and S418 is altered to render the SH3 domain inaccessible to binding N-WASp or other proteins. Phosphorylation of S405/418 results in liberating the SH3 domain, where it in turn is capable of binding and stimulating N-WASp activation. Expression of cortactin S405/418D phosphomimetic forms in cells increases branched actin networks in actin tails associated with cytoplasmic vesicles, providing support for this model in promoting cellular actin polymerization [47]. Initial assessments of cortactin structure by rotary shadow electron microscopy revealed cortactin to exist as a rod shaped monomer 220Å in length [76]. However, a recent biophysical analysis utilizing chemical crosslinking and small angle x-ray scattering suggests that cortactin exists in a more globular form, with the carboxyl terminal HP and SH3 domains folding back onto the amino terminal actin binding region [77]. Such a structure would support a “closed” conformation, although “open” structures were not observed, nor were the effects of ERK1/2 phosphorylation evaluated. Additional evidence for an inhibitory function of the amino terminus can be inferred from the ability of the cortactin carboxyl terminal domain to promote N-WASP-dependent cell motility as effectively as wild type cortactin [78], as well as the prevalence of the 85kDa form in invasive colorectal cancer [66].

In the ERK-Src “switch” model proposed for cortactin regulation, cortactin phosphorylation by Src at Y421, 470 and 486 serves to downregulate N-WASp activity promoted by S405/418 phosphorylation [46]. This model therefore suggests that serine and tyrosine phosphorylation of cortactin function in a reciprocal manner to govern N-WASp activation [58]. Our data with site-specific phosphorylation antibodies on lysates from EGF-stimulated cells indicates that S405/418 and Y421 are co-phosphorylated, and analysis of point mutant cortactin constructs does not indicate a reciprocal influence between cortactin serine and tyrosine phosphorylation events. These data suggest that cortactin function is not exclusively regulated by a serine-tyrosine “switch” mechanism. This view is additionally reinforced by the presence of pS418 and pY421 cortactin within lamellipodia and invadopodia. While our data do not rule out scenarios where such a mechanism may be employed at the cellular level, they are consistent with biochemical and cellular evidence indicating that tyrosine phosphorylation promotes N-WASp activity

through binding of the adaptor Nck1 [37,39], a component that was not present in the original assays where the “switch” mechanism was defined. The ability of cortactin to be simultaneously phosphorylated at S405/418 and Y421/470/486 may therefore provide cells with the ability to fine-tune the level of N-WASp activation and subsequent actin remodeling in response to diverse upstream stimulatory input that triggers motility and invasion.

Consistent with the mechanistic descriptions above, inhibition of carcinoma cell motility by MEK inhibition and S405/418A expression indicates that S405/418 cortactin phosphorylation is important in promoting and maintaining cell migration. While similar results were observed in wound healing assays with pancreatic cancer cells [47], our work extends these findings by evaluating the effects of pS405/418 on lamellipodia dynamics. The inability of MTLn3 cells expressing S405/418A cortactin to maintain EGF-stimulated dominant lamellipodia persistence implies that the actin networks within these cells fail to maintain proper Arp2/3 nucleation, or are more labile following lamellipodia extension. While N-WASp activation and Arp2/3-mediated actin polymerization resultant of cortactin SH3 domain binding has been shown to be important in governing motility in multiple cell types [47,78], a detailed study of EGF-induced lamellipodia protrusion in this cell type has recently shown that WAVE2 and formin proteins, not N-WASp, are responsible for lamellipodia protrusion [14]. These results would therefore rule out a role for direct N-WASp activation by pS405/418 cortactin in MTLn3 lamellipodia extension. In addition to N-WASp, the cortactin SH3 domain interacts with several other proteins that have the potential to directly or indirectly regulate lamellipodia actin dynamics (reviewed in [62]). In particular, cortactin binds and activates the Dbl family guanine nucleotide exchange factor faciogenital dysplasia protein 1 (FGD1) [79,80], a potent activator of Cdc42 [50]. Cdc42 activity is required for localization of WAVE2 and its activator IRSp53 to the cell membrane, where it mediates lamellipodia extension [81]. FGD1 also activates the MEK-ERK1/2 pathway [50], allowing the potential of a positive feedback loop in stimulating cortactin S405/418 phosphorylation through continuous cortactin SH3-mediated FGD1 activity. FGD1 binding represents just one possible cortactin SH3 domain ligand with the capability to influence WAVE2 localization and lamellipodia dynamics. Whether such an

FGD1-based regulatory circuit or other modes of potential pS405/418 cortactin regulation of WAVE2 activity exist in MTLn3 cells remains to be confirmed.

Previous studies on lamellipodia dynamics in other cells types indicate that cortactin removal decreases lamellipodia persistence, which can be rescued by re-expression of a cortactin amino terminal fragment lacking the carboxyl terminal region [20], eliminating contributions from pS405/418 in this system. These results differ from our work in MTLn3 cells, where cortactin removal results in enhanced persistence that can be rescued by re-expression of wild type cortactin. It is likely that these observed differences are due to a combination of different cell types, chemotactic cues, and analysis of dominant, initial lamellipodia versus steady-state lamellipodia dynamics [62]. Interestingly, inhibition of ERK1/2 signaling during macrophage lamellipodia extension results in decreased lamellipodia stability, with similar kymograph profiles to EGF-stimulated MTLn3 cells with S405/418A expression [64]. These studies provide supporting evidence for our observations.

Through the use of phosphorylation-specific antibodies, we have analyzed the localization of cortactin pS405 and pS418 in tumor cells and tissue, as well as the signaling pathways regulating pS405/418 phosphorylation. Through the use of these reagents, we have been able to validate and further clarify the role of pS405/418 in cortactin-based signaling. Our functional studies of carcinoma motility and lamellipodia dynamics with phosphorylation-null constructs have shed additional light on the role these phosphorylation events play in regulating lamellipodia function involved in tumor cell movement.

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## **Author Contributions**

Conceived and designed the experiments: LCK, KEH, AGA, SAW. Performed the experiments: LCK, KEH, AGA. Acquired and analyzed the data: LCK, KEH, AGA, KHM. Contributed reagents/materials/analysis tools: KHM, SAW. Wrote the paper: LCK, KEH, AGA, SAW.

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## **Figure Legends**

**Figure 1** Specificity and validation of pS405 and pS418 phospho-specific cortactin antibodies. **(A)** Phospho-specific recognition of anti-cortactin pS405 and pS418 antibodies. Clarified lysates (50µg) from 1483 cells transfected with Myc-tagged wild-type cortactin (WT), Myc-cortactin S405A, Myc-cortactin S418A or Myc-cortactin S405A,S418A point mutants were immunoblotted with affinity purified anti-Cort-pS418 (left) and anti-Cort-pS405 (right) antibodies. **(B)** Localization of pS418 cortactin in areas of motile and invasive actin dynamics. UMSSC2 cells (*top row*) were serum starved for 16h prior to stimulation with 100ng/ml EGF for 1 h to induce lamellipodia formation, while UMSSC1 cells (*middle row*) were plated on FITC-conjugated gelatin coated coverslips (pseudocolored white) for 6 h to promote invadopodia formation. Cells were fixed, permeablized, and labeled with TRITC-phalloidin (Actin), anti-cortactin (Cort) and anti-cortactin-pS418 antibodies. Arrows denote localization of pS418 cortactin with total cortactin and F-actin in lamellipodia (*top*) and to invadopodia (*middle*) coinciding with areas of active matrix degradation. Bottom panels are magnified views of the indicated cellular region. Bars, 10µm. **(C)** Localization of pS418 cortactin in HNSCC tumor tissue. Serial sections from a patient with invasive HNSCC were processed for immunohistochemistry with control IgG (Ctl), pS418 cortactin (pS418), total cortactin (Cort) and phospho-ERK1/2 (pERK) antibodies. Sections were counterstained with hematoxylin. Arrowheads indicate areas of peripheral pS418 cortactin and total cortactin enrichment. Bar, 100µm.

**Figure 2** Growth factor-stimulated Erk 1/2 activation mediates phosphorylation of cortactin at serine 405 and 418. **(A)** Growth factor-induced phosphorylation of cortactin S405 and S418. Serum starved UMSSC1 cells were stimulated with EGF (left) or FBS (right) for the indicated times. Cells were lysed and analyzed by Western blotting with anti-Cort-pS418 and anti-Cort-pS405 antibodies. Blots were stripped and reprobed with a pan-cortactin antibody to confirm equal loading (*bottom*). **(B)** Pharmacologic MEK inhibition inhibits cortactin S405 and S418 phosphorylation. UMSSC1 (left) and 1483 (right) cells were serum starved in the presence of the indicated selumetinib concentrations prior to stimulation with EGF for 20 min. Cortactin immunoprecipitated

from cell extracts was assayed by Western blotting with anti-Cort- pS418 and anti-Cort-pS405 antibodies. Blots were stripped and reprobed with pan- cortactin antibody as in (A) (*bottom panels*). Selumetinib efficacy was verified by the blotting of lysates from selected timepoints with phospho-ERK1/2 (pERK1/2) and pan ERK1/2 antibodies (*bottom*). All blots are representative images from 3-4 independent experiments.

**Figure 3** EGF-induced conversion of cortactin from 80kDa to 85kDa is impaired by Src and MEK1/2 inhibition. EGF induces the p80kDa to p85kDa shift in HNSCC cells. Serum starved UMSCC2 **(A)** and 1483 **(B)** cells were treated with 100ng/ml EGF for the indicated times. Clarified lysates were assayed by Western blotting with anti- cortactin, anti-Cort-pS418, anti-cort-pS405, anti-Cort-pY421, anti-Src-pY418, anti-pErk1/2 and total Erk1/2 antibodies as indicated. Red bars denote the position of the 85 kDa cortactin form; black bars denote the 80 kDa form. **(C)** Inhibition of Src and MEK1/2 kinase activity inhibits the cortactin “shift”. UMSCC2 cells were treated with vehicle (DMSO), saracatinib, or selumetinib for 16 h in serum free media. Cells were stimulated with 100ng/ml EGF for 1 h, lysed and analyzed by Western blot analysis with an anti-cortactin antibody.

**Figure 4** Cortactin tyrosine and serine phosphorylation resultant of v-Src activation are not interdependent. **(A)** Schematic diagram of the cortactin point mutant constructs assayed for phosphorylation. Mutated codons are denoted on the left and displayed with the corresponding mutant amino acid at the appropriate position within cortactin in red. **(B)** Murine fibroblasts lacking endogenous Src, Yes and Fyn (SYF) were transfected with murine-specific cortactin siRNA and cultured for 48 h to deplete endogenous cortactin. Cells were subsequently co-transfected with the temperature-sensitive v-Src construct La29 (*tsLa29*) and wild-type or the indicated myc- tagged human cortactin point-mutant constructs at 41°C (non-permissive temperature). TPM; triple point mutant consisting of Y-F mutations at positions 421, 470 and 486. After transfection, cells were cultured at 41°C, then shifted to 35°C (permissive temperature) for 2 h to promote v-Src activation. Recombinant cortactin proteins were assayed by immunoblotting with anti-cortactin-pY421, anti-cortactin-pS405, anti-myc, anti-cortactin, and anti-β-actin antibodies. Note that the inability of cortactin to be phosphorylated on



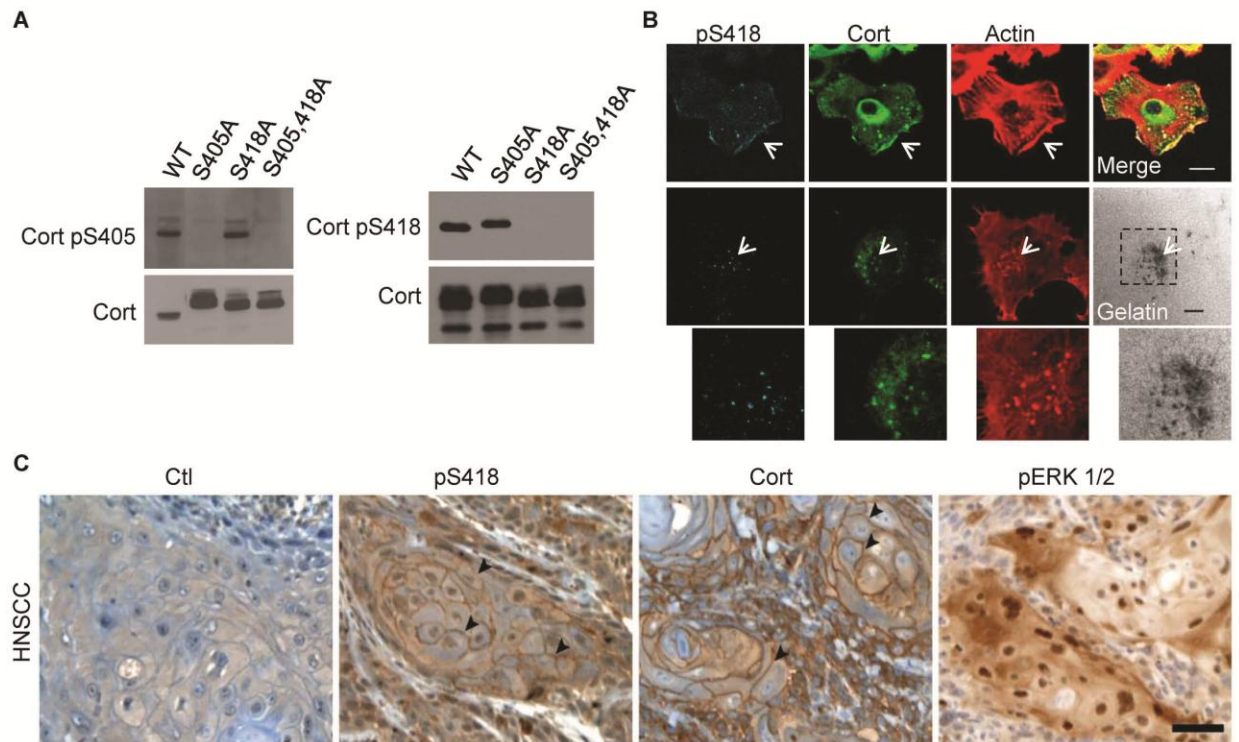
Y421 does not impact its ability to be phosphorylated on S405, nor does lack of S405 phosphorylation impact Y421 phosphorylation.

**Figure 5** Targeted inhibition of MEK1/2 inhibits HNSCC cell motility. 1483 and UMSCC1 cells ( $5 \times 10^5$ ) were starved for 24 h in the presence of vehicle (DMSO) or increasing concentrations of selumetinib as indicated. Cells were assayed for motility by electric substrate impedance sensing (ECIS) following stimulation with complete media containing the matched selumetinib concentration for 24 h. Data is displayed as slope values calculated from the linear part of ECIS tracings. Bars represent mean  $\pm$  SE. \*,  $p < 0.05$  compared to DMSO treated control cells.

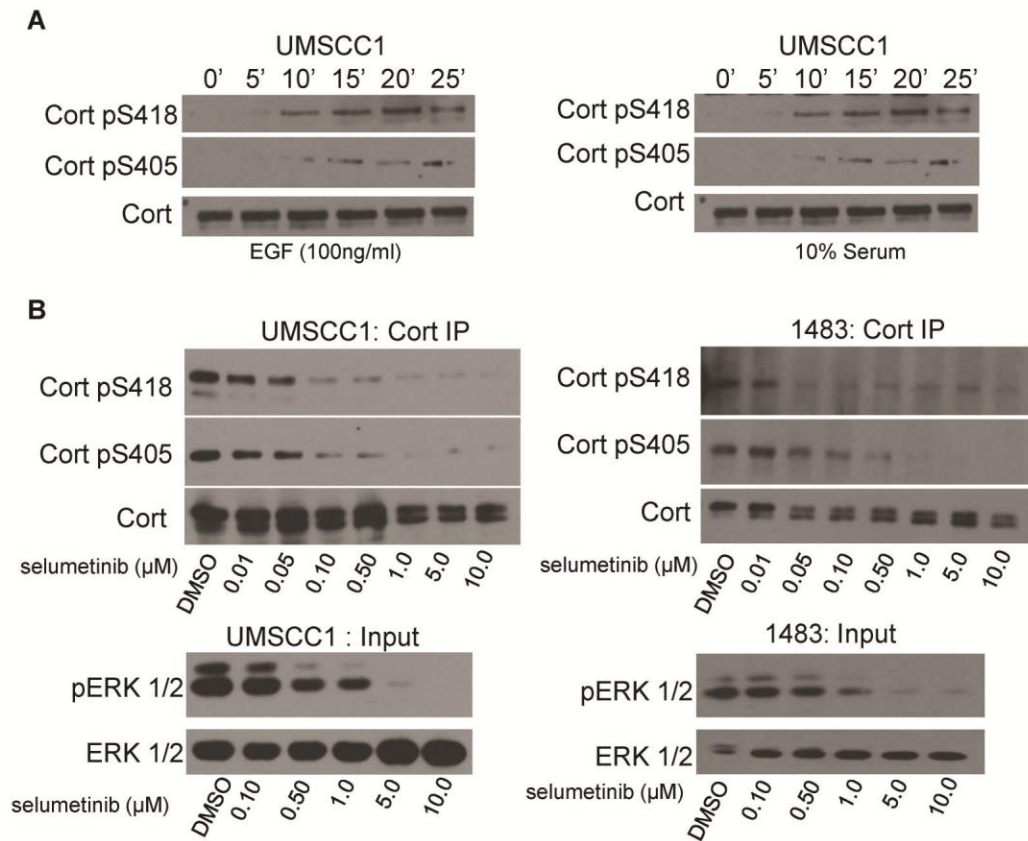
**Figure 6** Cortactin phosphorylation at serine 405 and 418 regulates carcinoma cell migration and adhesion. **(A)** Expression of GFP-cortactin constructs in MTLn3 cells. MTLn3 cells were transfected with murine-specific cortactin siRNA (Si) for 48 h to silence endogenous cortactin expression. Cells were subsequently transfected with the indicated human GFP-tagged cortactin wild-type and the various Erk1/2 phosphorylation-null point mutant constructs. Following transfection, cell lysates were immunoblotted with anti-cortactin, anti-GFP and anti- $\beta$ -actin antibodies. Solid arrowheads indicate the position of GFP-tagged cortactin variants; open arrowheads denote the position of endogenous cortactin. **(B)** Serine 405 and 418 phosphorylation is required for efficient carcinoma cell motility. MTLn3 cells transfected as in (A) were analyzed for cell migration by ECIS. Cell impedance versus time plots for each transfected line are shown on the *left*; slope values calculated from the linear region of each plot are displayed on the *right*. **(C)** Carcinoma cell spreading requires phosphorylation of cortactin S405 and S418. Transfected MTLn3 cells were plated, with rates of spreading were monitored by ECIS tracings over time *left*. Slope values from the linear regions are shown on the right. Bars represent mean  $\pm$  SE for 3 independent experiments. \*,  $P < 0.05$  compared to control (ctl) cells.

**Figure 7** Cortactin phosphorylation at serine 405 and 418 is required for lamellipodia persistence. **(A)** Kymographic analysis of MTLn3 lamellipodia. Serum starved MTLn3 cells (Ctl) or cells transfected with the indicated cortactin siRNA and cortactin constructs were monitored for dominant lamellipodia formation by live cell imaging following EGF

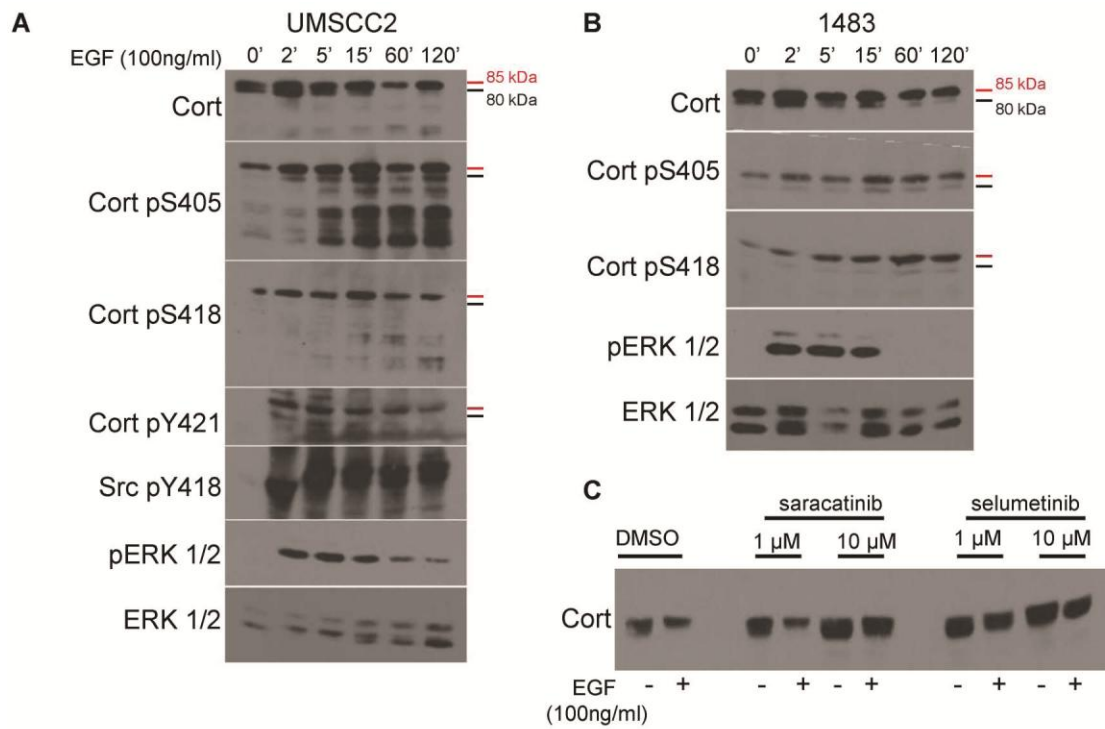
stimulation. Quantification of lamellipodia protrusion rates, length of extension, and time of lamellipodia persistence are shown for each experimental condition.  $\geq 10$  cells were analyzed for each group from  $\geq 3$  independent experiments. **(B)** Representative kymograms of each cell type. Kymograms were constructed from 1-pixel wide lines drawn from the initial leading edge and in the direction of the dominant lamellipodia. Cells were visualized by fluorescent microscopy using mCherry- $\beta$ -actin as the lamellipodia marker. Images were captured every 5 sec for a period of 15 min. Black lines denote the baseline position of the leading edge prior to EGF stimulation. Bar; 5  $\mu$ m.



Kelley et al., Fig 1

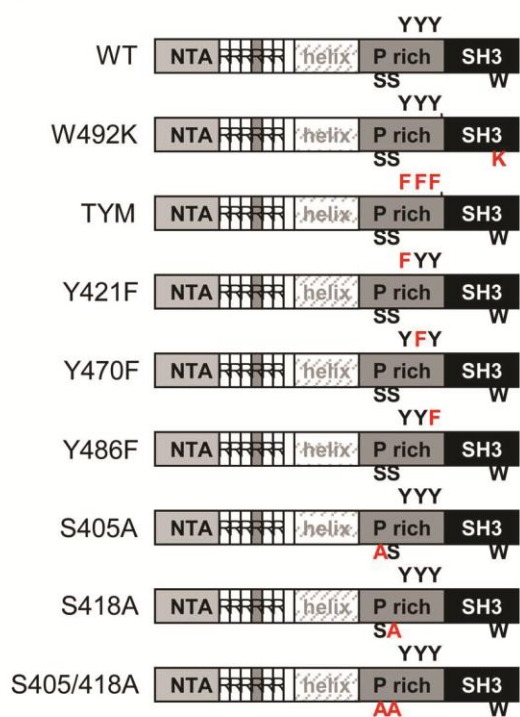


Kelley et al., Fig 2

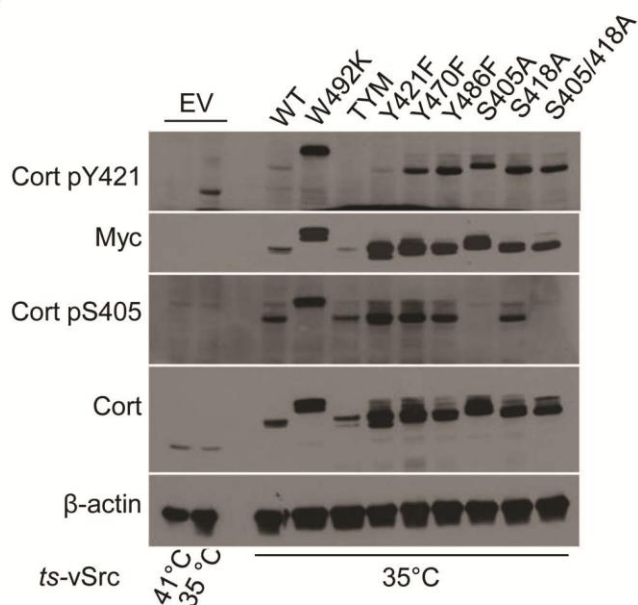


Kelley et al., Fig 3

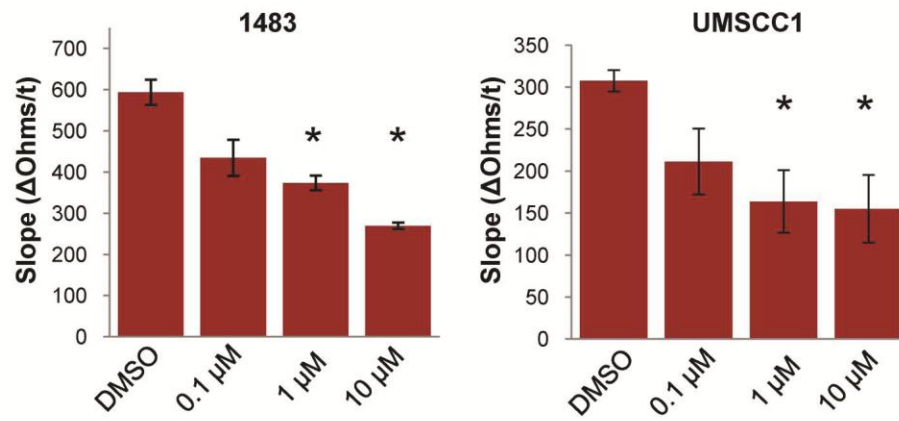
**A**



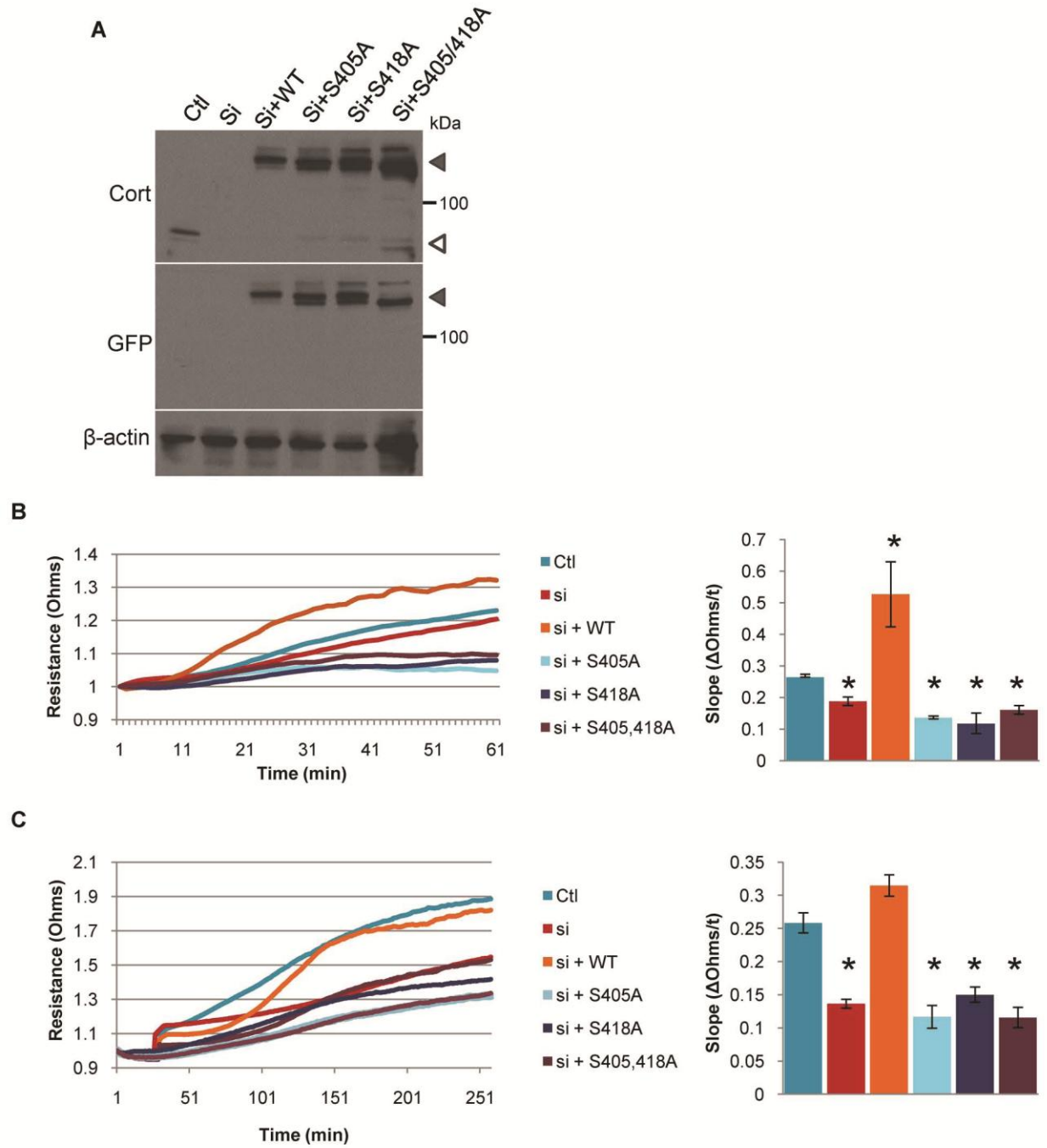
**B**



Kelley et al., Fig 4

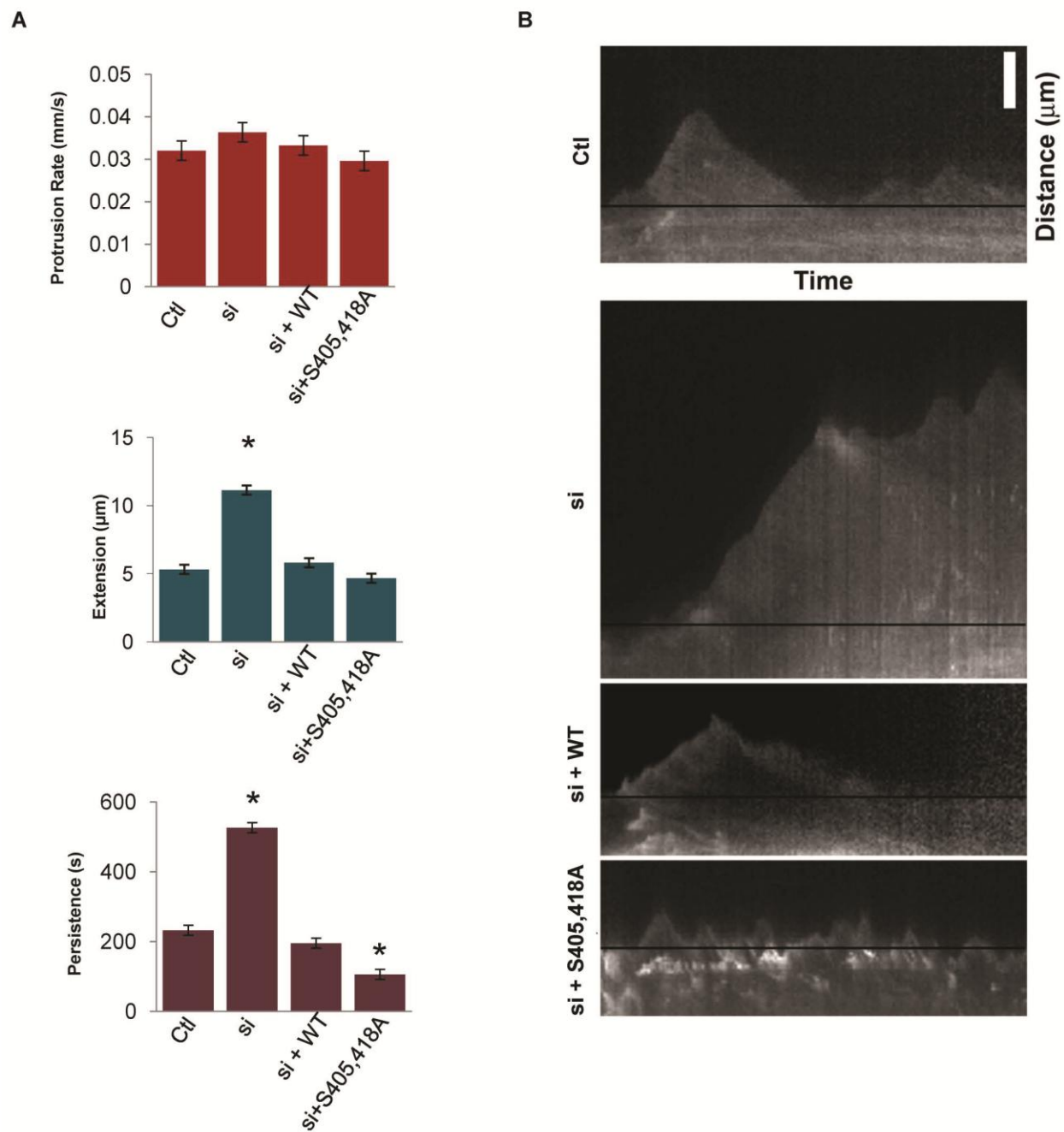


Kelley et al., Fig 5



Kelley et al., Fig 6





Kelley et al., Fig 7

## **General Discussion**

The successful treatment of CML and GIST patients with imatinib makes it an attractive chemotherapeutic agent for solid tumor types with elevated Abl activity (1-5). Unfortunately, single agent imatinib phase I and II trials have demonstrated little benefit for breast or pancreatic cancer patients. Furthermore, imatinib treatment of prostate, NSCLC, and HNSCC patients in clinical trials had deleterious effects, resulting in enhanced tumor progression (6-10,10-12). The poor response rate for chemotherapy targeting Abl in solid tumors may be attributed to the consequential oncogenic effects of Abl inhibition in these specific diseases.

On one hand, Abl family kinases enhance tumor proliferation and invasion in breast, melanoma, gastric and NSCLC (1,1-5,13-16). Conversely, Abl family kinases have also been shown to inhibit TGF $\beta$  induced EMT, tumor growth, and/or motility in breast cancer. Moreover, thyroid cancer cells treated with imatinib results in enhanced cell migration. *In vivo*, imatinib treatment increases breast cancer tumor size in xenograft mouse models, and; *in vitro* analysis of imatinib treated MA-11 breast cancer cells demonstrated enhanced migration, elevated Erk 1/2 and Wnt signaling (17-20). Study 1 provides additional insight into the divergent roles of Abl in tumor invasion by showing that Abl regulates invasive signaling in a tumor type specific manner (21). This work confirmed the published pro-invasive Abl family kinases activity in breast cancer invadopodia formation and 3-D collagen invasion utilizing the common MDA-MB-231 cell line (4,5), as well as demonstrating an opposing role for Abl in HNSCC invasion where Abl functions an invasion suppressor.

Study 1 demonstrates elevated basal activation of Src and EGFR in HNSCC compared to MDA-MB-231 cells. Elevated Src activation in HNSCC can regulate ECM degradation and invasion by bypassing Abl family kinase regulation, contrary to the proposed EGFR/Src/Arg/Cortactin-mediated regulation of invadopodia maturation published by the Koleske group (4). In HNSCC, Src activity is responsible for the increased ECM-degradative activity of invadopodia induced by inhibition of Abl kinase activation by imatinib or RNAi. The regulatory role of Src in HNSCC was further examined in Study 2. The efficacy of the dual Src/Abl inhibitor saracatinib was

demonstrated in HNSCC cell lines by Western blot analysis of Src substrate phosphorylation sites in FAK, p130Cas and cortactin. Study 2 confirmed the regulatory role of Src by the inhibition of invadopodia formation and activity, matrigel invasion and metastasis *in vivo*. Interestingly, analysis of CML cell lines resistant to the Abl-specific inhibitor nilotinib with overexpression of the Src-family member Lyn regained sensitivity to nilotinib when Lyn was removed by RNAi (22,23). Similar results were observed with imatinib-resistant human GIST xenograph models, where resistant tumors had integrin-mediated elevated Src and Lyn activation (24). Collectively, these reports and the studies herein indicate that elevated Src kinase activity supersedes any pro- or anti-invasive role governed by Abl. In addition, these studies suggest the activation of Src resultant from Abl inhibition observed in Study 1 is not specific to HNSCC.

To determine the molecular mechanism for imatinib-mediated activation of Src in HNSCC and subsequent enhanced invadopodia proteolytic activity and invasion, we examined the activation of EGFR and Erk 1/2, two key components of invadopodia regulation. In Study 1, EGFR and Erk 1/2 activity were elevated in imatinib-treated HNSCC, but not in MDA-MB-231 cells. The increased Erk1/2 phosphorylation achieved with Abl family kinase inhibition is contrary to current dogma, where the RAS/RAF/MEK/Erk signaling pathway is activated by either BCR-Abl or integrin-mediated c-Abl activity (25-27). Unexpectedly, increased phosphorylation of Erk 1/2 is frequently observed in imatinib-resistant CML cells and other tumor types. CD34+, BCR-Abl+ progenitor leukemic cells treated with imatinib or nilotinib results in enhanced Erk 1/2 activation (28). Imatinib also stimulates elevated phosphorylation of Erk 1/2, PI3K/Akt, and Stat3 in PDGFR $\beta$  expressing glioblastoma cells (29). Conversely, imatinib treated colon adenocarcinoma cells display apoptosis and disrupted F-actin cytoskeletal networks (30). Such studies further highlight the need for better insight into the mechanisms utilized by tumors to acquire imatinib or nilotinib resistance if these compounds are to be considered for widespread use in solid tumors, as well as for current treatment of CML and GIST patients where imatinib and nilotinib forms an important part of the standard of care for these individuals.

Along these lines, Packer et. al. have examined all known components of the RAS/RAF/MEK/Erk1/2 signaling pathway in an imatinib-resistant CML cell line, determining that imatinib induces activation of RAF and MEK in addition to Erk1/2. Cells lines from solid tumors were compared that contained constitutive inactivation of this pathway due to upstream KRAS mutation. When treated with imatinib, nilotinib or dasatinib, the RAF isoform BRAF was inhibited, but the related form CRAF was partially activated by heterodimerization with inactive BRAF, presenting a possible mechanism for imatinib activation of Erk1/2 (31). This is supported by the observation of CRAF hyperactivation in melanoma and thyroid cancer patients containing inactivating BRAF V600E mutations (32). MDA-MB-231 cells contain an activating KRAS mutation that hyperactivates KRAS/RAF/MEK/Erk1/2 and potentially diminishes the effect of imatinib-induced CRAF activation. This creates a plausible explanation for divergent effects observed with imatinib treatment between our HNSCC cell lines (UMSCC1 and OSC19) and MDA-MB-231. However, this mechanism does not explain the increased ECM degradation observed in UMSCC1 cells with Abl knocked-down seen in Study 1.

Elevated expression and secretion of HB-EGF in imatinib-treated HNSCC cell lines was shown by our laboratory (Study 1) and others (33), presenting an alternate mechanism for imatinib-mediated induction of EGFR, Src, and Erk 1/2 activity. Nevertheless, HB-EGF is likely not the only cytokine up-regulated in HNSCC with imatinib treatment. Imatinib exposure enhanced ephrin and osteonectin in BCR-Abl<sup>+</sup> leukemic cell lines (34,35).

Cortactin is overexpressed in HNSCC and other cancers and correlates with poor prognosis (36-40). Elevated cortactin tyrosine phosphorylation is associated with increased invasion and metastasis (13,14,21,41-46). Cortactin is also phosphorylated by Erk 1/2 at serine sites 405 and 418, but little was known of the effects of cortactin serine phosphorylation on invasion and metastasis (47). Martinez-Quiles et al demonstrated cortactin serine phosphorylation increased N-WASp binding to the SH3 domain of cortactin, enhancing cortactin-mediated activation of Arp 2/3 actin polymerization (48). Study 3 further elucidated the effect of cortactin phosphorylation by Erk 1/2 on cancer cell motility and lamellipodia function. Serine 405/418

phosphorylation resulted in increased adhesion and motility with in EGF-stimulated HNSCC cells. This motility enhancement can be attributed in part to an increase in lamellipodia persistence. Our laboratory (Study 3) and others have shown that cortactin serine phosphorylation and interaction with p120 catenin are essential for lamellipodia persistence and lamellipodial-mediated ECM recycling (49,50). Cortactin serine phosphorylation by Erk 1/2 also modulates invadopodia formation and proteolytic activity (51). These results collectively support a role for Erk 1/2 phosphorylation of cortactin in regulating motility structures utilized for amoeboid, mesenchymal and collective cell motility and invasion (52).

Imatinib treatment enhances Erk 1/2, Src, and/or EGFR phosphorylation in HNSCC, CML and other tumor types. Our laboratory and others have demonstrated that imatinib can increase or decrease invasion and metastasis by activating or inhibiting key components of these processes- namely Erk1/2, Src, and EGFR. Abl appears to be a key component in mediating these divergent effects and can function as an anti- or pro-oncogenic protein dependent on cellular context. The regulating mechanism(s) that modulate the dichotomous roles of Abl in tumor motility are currently unknown. An understanding of this complex process is crucial as the use of imatinib expands to solid tumors in the clinic in order to prevent detrimental effects to patients undergoing imatinib treatment.

Imatinib is the first successful targeted kinase inhibitor that moved from the bench to the clinic, ushering in the era of modern targeted therapeutic intervention in cancer. The efficacy of these targeted therapies has been mixed, from the success of imatinib to the puzzling lack of response with dasatinib in phase II and III trials where preclinical work showed great promise. Why certain targets are more effective than others when therapeutically inhibited is not fully understood, nor are the compensatory mechanisms tumor cells utilize to overcome targeted therapies in developing drug resistance.

Drug resistance has been attributed to three main reasons; the heterogeneity of tumors that enable a subset of tumors cells (likely tumor initiating stem cells) to survive chemotherapeutic agents, the complexity of the microenvironment (cellular and non-cellular) providing a protective niche for the tumor cells; and tumor cell plasticity (53).

Tumor plasticity allows the tumor to reversibly change and adapt their phenotype to intrinsic and external cues without permanently altering genomic DNA. EMT confers stem cell like properties and chemoresistance in tumor cells. Transcription factors such as Zeb1, Twist, and Snail are key components that regulate EMT and are current areas of intense focus in efforts to reverse molecular and phenotypic elements of transformation (54). However, it is clear that EMT is not the sole factor regulating tumor plasticity (55), as our work and others indicates that adaptive kinase responses play important roles in mediating drug resistance (21,28,29). Duncan et. al. utilized an innovative multiplexed inhibitor beads (MIBs) system that binds over 60% of the kinase kinome to examine the rapid kinome reprogramming induced by MEK inhibitor treatment of triple negative breast cancer (56). MIBs are generated by immobilizing several broad kinase inhibitors to sepharose beads. MIBs selectively bind active kinases due to the general mechanism utilized by kinase inhibitors, blocking the ATP binding sites; this allows the monitoring of altered activity of over a hundred kinases simultaneously (56). Our work demonstrates kinase “reprogramming” within hours after drug treatment enhances HNSCC metastasis and invasion making imatinib an interesting candidate for this process. The dichotomous functions of Abl in different cancer types suggest that it may play a key regulatory role in governing tumor plasticity responsible for facilitating drug resistance.

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## **Appendix**

Video Article

# Quantitative Measurement of Invadopodia-mediated Extracellular Matrix Proteolysis in Single and Multicellular Contexts

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URL: <http://www.jove.com/video/4119/>

DOI: 10.3791/4119

Keywords: Cellular Biology, Issue 66, Cancer Biology, Anatomy, Molecular Biology, Biochemistry, invadopodia, extracellular matrix, gelatin, confocal microscopy, quantification, oregon green

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## Abstract

Cellular invasion into local tissues is a process important in development and homeostasis. Malregulated invasion and subsequent cell movement is characteristic of multiple pathological processes, including inflammation, cardiovascular disease and tumor cell metastasis<sup>1</sup>. Focalized proteolytic degradation of extracellular matrix (ECM) components in the epithelial or endothelial basement membrane is a critical step in initiating cellular invasion. In tumor cells, extensive *in vitro* analysis has determined that ECM degradation is accomplished by ventral actin-rich membrane protrusive structures termed invadopodia<sup>2,3</sup>. Invadopodia form in close apposition to the ECM, where they moderate ECM breakdown through the action of matrix metalloproteinases (MMPs). The ability of tumor cells to form invadopodia directly correlates with the ability to invade into local stroma and associated vascular components<sup>3</sup>.

Visualization of invadopodia-mediated ECM degradation of cells by fluorescent microscopy using dye-labeled matrix proteins coated onto glass coverslips has emerged as the most prevalent technique for evaluating the degree of matrix proteolysis and cellular invasive potential<sup>4,5</sup>. Here we describe a version of the standard method for generating fluorescently-labeled glass coverslips utilizing a commercially available Oregon Green-488 gelatin conjugate. This method is easily scaled to rapidly produce large numbers of coated coverslips. We show some of the common microscopic artifacts that are often encountered during this procedure and how these can be avoided. Finally, we describe standardized methods using readily available computer software to allow quantification of labeled gelatin matrix degradation mediated by individual cells and by entire cellular populations. The described procedures provide the ability to accurately and reproducibly monitor invadopodia activity, and can also serve as a platform for evaluating the efficacy of modulating protein expression or testing of anti-invasive compounds on extracellular matrix degradation in single and multicellular settings.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/4119/>

## Protocol

### 1. Production of Oregon Green 488-gelatin Coated Coverslips

1. Prepare an unlabeled 5% (w/w) stock gelatin/sucrose solution by adding 1.25 g gelatin and 1.25 g sucrose in PBS to a final volume of 50 ml. Warm the stock gelatin solution to 37 °C and ensure it is entirely melted before use. Store the final mixture at 4 °C.
2. Clean 13 mm diameter #1 glass coverslips by placing an individual coverslip into each well of a 24 well plastic tissue culture plate. Add 500 µl of 20% nitric acid to each well and incubate for 30 min. Aspirate the nitric acid solution and wash coverslips three times with deionized water.
3. Coat coverslips with 500 µl of 50 µg/ml poly-L-lysine (prepared from 0.1% stock solution and diluted in deionized water) to each well for 20 min at room temperature. Aspirate the solution and wash three times with PBS. Poly-L-lysine coating facilitates even coating and bonding of the overlying labeled gelatin.
4. Add 500 µl of 0.5% glutaraldehyde (made fresh before use) to each well and incubate the 24 well plates on ice for 15 min. Aspirate and wash three times with cold PBS. Be sure to remove all traces of PBS prior to gelatin coating. Keep plates on ice during all washes until gelatin is added.
5. Reconstitute the Oregon Green 488-conjugated gelatin as per manufacturer's protocol and warm it and the unlabeled 5% gelatin/sucrose solution from (1.1) to 37 °C. Dilute one part Oregon Green 488 gelatin into eight parts of unlabeled gelatin/sucrose (*i.e.*; 500 µl of Oregon Green 488 gelatin into 4 ml of 5% gelatin mixture). Pipet 100 µl of the diluted 488-gelatin mixture (kept at 37 °C) onto each coverslip, using enough gelatin to coat the coverslip without manual spreading (which can lead to uneven coverslip coating as shown in **Figure 3B**). It is important to keep the diluted 488-gelatin mixture at 37 °C during the coating procedure to prevent premature solidification. From this step forward the coverslips should be kept in the dark as much as possible to avoid potential photobleaching. Other ECM proteins conjugated to different fluorophores can be substituted for Oregon Green 488 gelatin (see Discussion).



- Once all coverslips are coated in a single plate, hold the 24 well plate at an angle and remove excess gelatin from each well by vacuum aspiration. Incubate coated coverslips in the dark for 10 min at room temperature.
- Wash the coverslips three times with PBS, then add 500  $\mu$ l of freshly made 5 mg/ml sodium borohydride ( $\text{NaBH}_4$ ) for 15 min at room temperature to reduce and inactivate residual glutaraldehyde. Sodium borohydride is effervescent, and small bubbles will be evident on and around each coverslip.
- Remove the  $\text{NaBH}_4$  solution by vacuum aspiration with a quick sweeping motion around the outside of each well. Take care not to pick up any floating coverslips that became detached from the bottom of the tissue culture plate during  $\text{NaBH}_4$  treatment. Detached coverslips that float to the top may be gently pushed back down to the well bottom, but care must be taken to avoid damaging the protein coating. Wash each well three times with PBS and then incubate coverslips in 70% ethanol for 30 min at room temperature.
- Using sterile technique, transfer the coverslip-containing plates to a type IIA/B cell culture laminar flow hood and rinse coverslips three times with sterile PBS. At this point coverslips can be stored in PBS protected from light at 4 °C for at least two months.
- Transfer coverslips to be used for degradation assays to an empty well of a new 24 well plate by careful removal using a sterile needle and forceps. Equilibrate coverslips for 1-24 hr with complete media appropriate to the specific cell type being assayed. Care must be taken not to invert the coverslip or scratch the gelatin coating (see **Figure 3B**).

## 2. Plating and Processing of Cells on Oregon Green 488-gelatin Coated Coverslips to Assay ECM Degradation

- Seed  $3\text{-}5 \times 10^4$  cells onto a coverslip within each well of the 24 well plate.
- Conduct a time course study to determine optimal times required for invadopodia degradation activity for the particular cell line/type of interest. Most invasive cells require a time between 4-24 h for degradation to become apparent, although this range can vary widely and should be empirically determined. To synchronize invadopodia activity, cells can be treated with MMP inhibitors (e.g., GM 6001) for a desired time period, then wash out the inhibitor to allow invadopodia activity to proceed (for example, see<sup>6</sup>).
- Rinse coverslips three times with PBS, then fix cells with 500  $\mu$ l of 10% buffered formalin phosphate for 15 min. Rinse three times with PBS and permeabilize for 4 min with 0.4% Triton X-100 in PBS. Rinse three times with PBS to remove the Triton X-100.
- Label cells using any standard protocol for immunofluorescence staining (see<sup>7</sup> for example) by co-labeling cells with fluorescent conjugated phalloidin to visualize actin filaments (F-actin) and for a known marker protein that localizes to invadopodia (e.g. cortactin<sup>5</sup>, TKS5<sup>8</sup>, or N-WASP<sup>9</sup>). Remember to avoid using 488-labeled secondary antibodies or GFP-labeled proteins if using Oregon Green 488 or FITC-labeled gelatin to prevent signal interference.
- Mount stained coverslips onto glass microscope slides by carefully inverting the coverslip and placing it on a drop of ProLong Gold antifade or similar reagent.
- To assess matrix degradation, image cells in appropriate channels using a conventional fluorescent or confocal microscope. Gelatin degradation is visualized as darker areas on the coverslip due to proteolytic removal of the fluorescent gelatin (**Figure 4A**). Labeling of cells for actin and an invadopodia marker protein allows for confirmation of invadopodia at sites of matrix degradation in merged images (**Figure 4A**).
- Degradation activity can also be monitored in real time by live cell imaging with fluorescent-tagged recombinant proteins to track invadopodia formation and matrix degradation<sup>5,10,11</sup>.

## 3. Quantification of Fluorescent Gelatin Degradation by Measuring Normalized Matrix Degradation

This analysis provides the normalized area of matrix degradation relative to the area of the cells or the number of cells. It is useful for analyzing entire microscopic fields of view where multiple cells are present that have been collectively treated with siRNA, growth factors or therapeutic agents. For this analysis, images collected at lower magnification are sufficient to efficiently collect information about populations of cells.

- Open the images in ImageJ<sup>12</sup>. ImageJ for microscopy can be downloaded from <http://www.macbiophotonics.ca/imagej/>.
- Check the scale information by choosing the menu command "Analyze/Set Scale." This information will import automatically with many file formats, but can be entered manually if required. Proper scaling is necessary to report measurements in microns rather than pixels.
- Select the appropriate measurements to track by choosing "Analyze/Set Measurements." Check *Area* and *Limit to Threshold*.
- Calculate the area of degradation using the fluorescent gelatin image (**Figure 5A**).
- Threshold the image ("Image/Adjust/Threshold") to set the upper and lower pixel intensity values to select the areas of degradation (highlighted in red; **Figure 5B**). In subsequent images, use the *Set* button in the Threshold window to set the same threshold for all images as an objective means to select degradation area.
- In some cases, the coverslip may not be perfectly flat when images are acquired. This causes the intensity of the gelatin to change across the image. If this variation creates problems when thresholding the image, correct for uneven illumination across the gelatin by subtracting the background ("Process/Subtract Background") or by filtering with a bandpass filter ("Process/FFT/Bandpass Filter") or a pseudo flatfield filter ("Process/Filters/Pseudo Flatfield") until the background intensity is uniform.
- Measure the area of matrix degradation ("Analyze/Analyze Particles"). In the Analyze Particles window, choose a particle size > 0 to remove noise from the selection. Show *Outlines* to identify regions of interest (ROIs). Check *Display Results* and *Summarize* to show measurements. If the drawing has specifically outlined all of the areas of degradation (**Figure 5C**), copy the Total Area measurement into a spreadsheet. If other objects were selected (such as debris), record only the areas of the relevant ROIs.
- Calculate the cell area using the phalloidin stained (F-actin) image (**Figure 5D**).
- Threshold the image ("Image/Adjust/Threshold") to set the upper and lower pixel intensity values so that the edges of the cells are selected (highlighted in red; **Figure 5E**). In subsequent images, use the *Set* button in the Threshold window to set the same threshold for all images as an objective means to select cell area.

10. Measure the area of the cells ("Analyze/Analyze Particles"). In the Analyze Particles window, choose a particle size > 0 to remove noise from the selection. Show *Outlines* to identify regions for analysis (Figure 5F). Check *Display Results* and *Summarize* to show area measurements. Do not check *Include Holes* if there are spaces between cells in a cluster so the non-selected pixels within the cluster will not be included in the cell area calculation. Choose *OK*.
11. Copy the *Area* results for relevant ROIs into a spreadsheet.
12. Calculate the area of gelatin degradation per total area of cells<sup>13</sup>.
13. An alternative approach would be to report the area of degradation per number of cells from counting nuclei (Figure 5G). This is necessary if manipulations alter the cell area between different compared treatment groups. Automatic counting works best if nuclei are well separated, uniform in intensity and round. Automatically count nuclei ("Plugins/Particle Analysis/Nucleus Counter"). Choose *Smallest and Largest Particle Size*, a *Threshold Method* and a *Smoothing Method*. Check *Subtract Background*, *Watershed Filter*, *Add Particles to ROI Manager* and *Show Summary* (Figure 5H).
14. If nuclei overlap extensively or have an irregular shape or texture, automatic counting may not produce an accurate count (Figure 5H, arrows on right). In this case, manual counting can be facilitated using the cell counter tool ("Plugins/Particle Analysis/Cell Counter"). This will keep count as cells are marked during a manual count (Figure 5I).
15. Copy the number of cells (nuclei) into a spreadsheet. Calculate the area of gelatin degradation per total number of cells.

#### 4. Quantification of Fluorescent Gelatin Degradation by Individual Cells in a Mixed Cellular Population

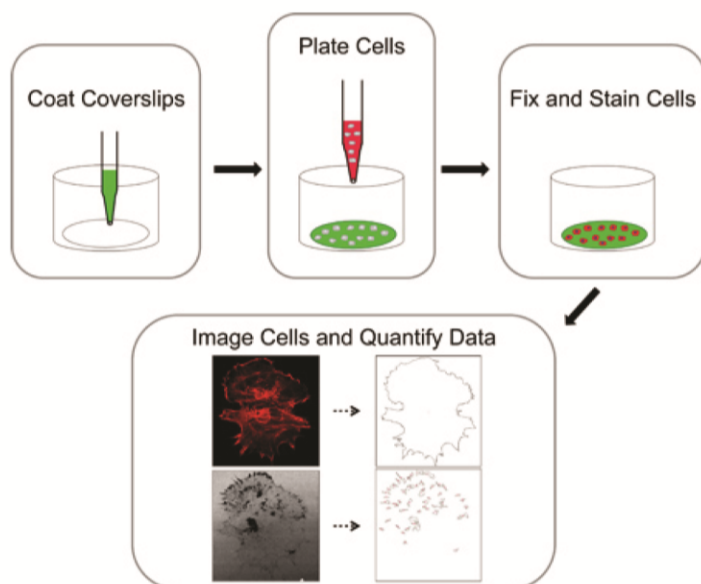
To evaluate matrix degradation resulting from specific cells in a population apart from other cells within the field (e.g., transfected versus non-transfected cells), the procedure in section 3 can be modified to measure the area of degradation under individual cells. An additional fluorescent channel is needed to mark transfected cells. In this instance, higher magnification images and well-separated cells are easier to quantitate.

1. Check the scale information by choosing the menu command "Analyze/Set Scale." Select the appropriate measurements to track by choosing "Analyze/Set Measurements." Check *Area* and *Limit to Threshold*.
2. For individual cells that are not touching, identify each cell using the F-actin image (Figure 6A). *Threshold* the image (see 3.9) (Figure 6B). It is important to capture the edges of the cells, but there can be holes inside that are not included in the threshold. Use the same intensity values across images to select cell boundaries.
3. To measure the area of the cells, use "Analyze/Analyze Particles." In the Analyze Particles window, choose a *Size* >0 (to eliminate noise), Show *Outlines*, and check *Display Results*, *Add to Manager* and *Include Holes* (to record the entire area inside the outline). Choose *OK* and record the *Area* for each cell from the Results window.
4. Identify which cells are transfected (Figure 6C).
5. Identify the areas of degradation using the fluorescent gelatin image (Figure 6D). If needed, filter the gelatin image to even background intensity (see 3.6). *Threshold* to select the areas of degradation, making note of the threshold settings (Figure 6E). On subsequent images, use these same upper and lower intensity values (using the *Set* button in the Threshold window) for an objective selection of areas of degradation.
6. Measure the areas of degradation under the cells. On the thresholded fluorescent gelatin image, show an outline of the cells by selecting ROIs in the ROI Manager window and selecting *Measure* (Figure 6F). Record the results and calculate the normalized area of degradation/cell or cell area.

#### 5. Representative Results

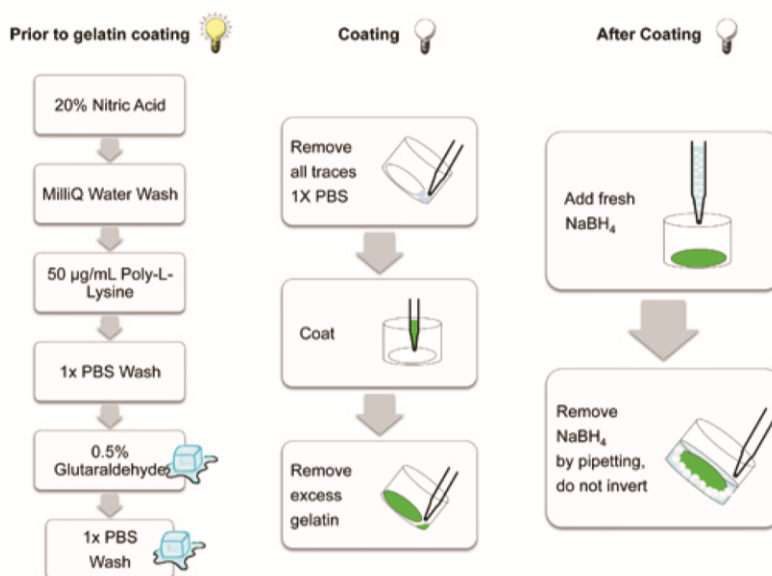
The overall schematic for the procedure is shown in Figure 1. The procedure entails preparation of glass coverslips and coating with fluorescently-conjugated gelatin, plating of cells onto the coated coverslips to allow cells to degrade the gelatin, fixing and labeling of cells for fluorescence microscopic analysis, imaging the fluorescent matrix to assess the matrix integrity, and objectively quantifying the degree of gelatin matrix degradation using computer software.





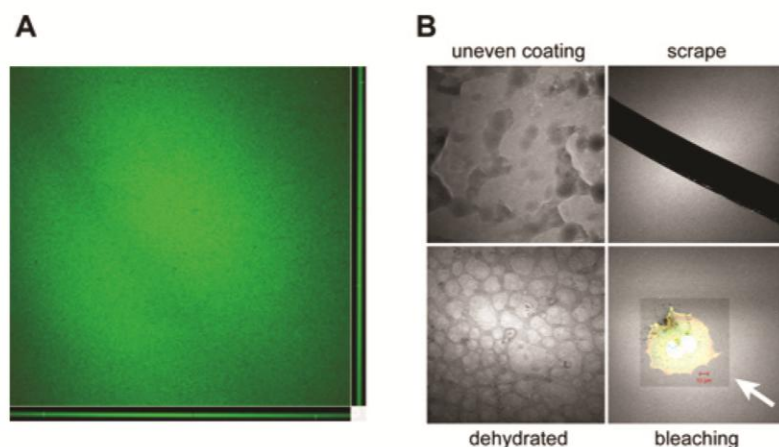
**Figure 1.** Overall schematic highlighting the key steps involved in fluorescent gelatin coating, cell plating, fixing and immunolabeling, and evaluating matrix proteolysis.

The key procedural steps involved in preparing and coating glass coverslips are outlined in **Figure 2**.



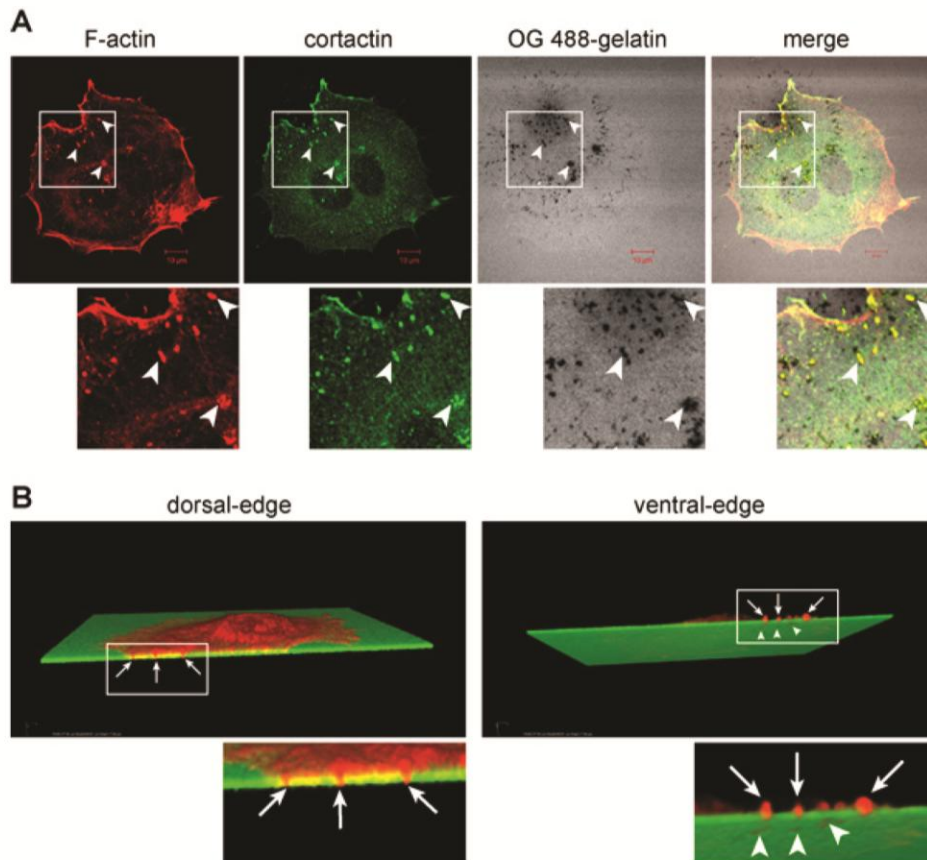
**Figure 2.** Schematic demonstrating the individual steps involved in preparing glass coverslips for gelatin matrix coating. Steps conducted in the light (lit bulb), on ice (cubes) and in the dark (non-illuminated bulb) are cartoon indicated. Steps conducted in the dark help prevent photobleaching of the fluorescent matrices.

When properly performed, coverslips are evenly coated with Oregon Green 488-conjugated gelatin, displaying homogenous fluorescence when visualized by microscopy (**Figure 3A**). Typical artifacts that can arise due to improper coating, handling, storage and usage of coated coverslips are shown in **Figure 3B**.



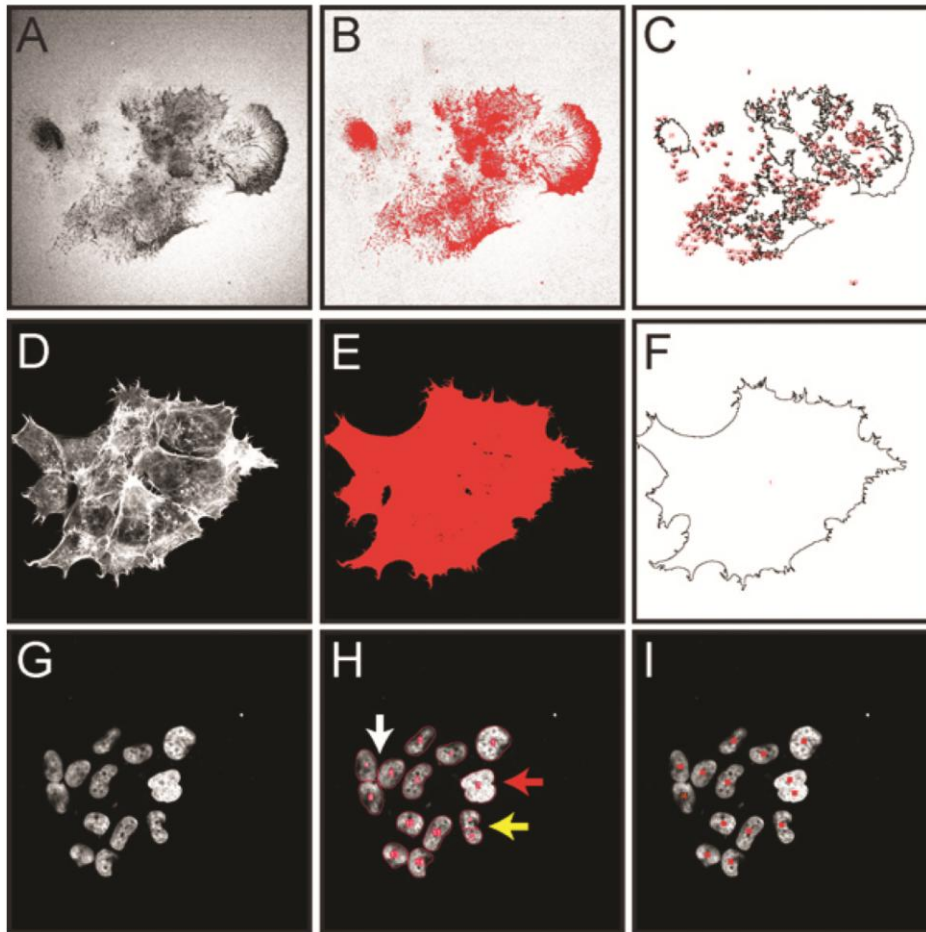
**Figure 3.** Examples of artifacts encountered during gelatin coated coverslip preparation and handling. **A.** Orthogonal view of a confocal z-stack showing the typical color and consistency of an Oregon Green 488-conjugated gelatin coated coverslip produced using the prescribed protocol. Coverslips should have a homogenous coating  $\sim 1\text{-}2\ \mu\text{m}$  thick as shown in the X-Z (*bottom*) and Y-Z (*right*) confocal planes. **B.** Artifacts that can occur during the coating and processing of gelatin-coated coverslips include: Improper covering of the coverslip during the coating process due to poor mixing, manual spreading or partial solidification of the gelatin mixture (*uneven coating*), removal of the coated matrix by scoring with needles or forceps during handling (*scrape*), drying of the coverslip surface during prolonged storage periods, resulting in a "cobblestone" appearance (*dehydrated*) and photobleaching of the fluorescent gelatin surface during imaging due to prolonged or high intensity light exposure (*bleaching*). White arrow indicates bleached area encompassing a plated OSC19 head and neck squamous carcinoma cell. The Oregon Green 488-conjugated gelatin is pseudocolored white to enhance image contrast. Bar,  $10\ \mu\text{m}$ .

The resulting thin matrices produced during this procedure provide a sensitive means to evaluate the ability of cells to degrade ECM. **Figure 4** demonstrates an example of invadopodia activity from an OSC19 cell plated on an Oregon Green-488 conjugated gelatin coverslip and imaged by conventional confocal microscopy as well as by volume-fill image rendering following three dimensional deconvolution.



**Figure 4.** Representative examples of invadopodia matrix degradation activity. **A.** Visualization of invadopodia and corresponding gelatin matrix proteolysis. OSC19 cells plated on Oregon Green 488-conjugated gelatin coverslips for 10 hr were fixed and labeled with rhodamine-conjugated phalloidin (F-actin) and anti-cortactin antibodies (visualized with an Alexa Fluor 647 secondary antibody and pseudocolored green). Invadopodia are evident as focal cytoplasmic concentrations of F-actin and cortactin that overlap with areas of gelatin clearing (dark holes in the matrix) within the merged image. Boxed regions containing arrowheads indicate individual invadopodia and areas of focal matrix proteolysis as shown in the enlarged regions below. Bar, 10  $\mu$ m. **B.** Volume fill visualization of invadopodia penetration into the ECM. OSC19 cells plated and stained as in (A) were visually rendered by obtaining 23 successive 0.32  $\mu$ m optical z-slices totaling 7.04  $\mu$ m for rhodamine-conjugated phalloidin and Oregon Green 488-conjugated gelatin. The native LSM file set for each channel was opened in AutoQuant X2.2 software and a 3D blind deconvolution of each image stack was performed using the recommended settings (10 iterations, medium noise). The processed images were saved as TIFF stacks that were then opened in NIS Elements and rendered as a volume view with alpha blending. The LUTs were adjusted, and a subvolume was created to show an edge inside the cell where invadopodia are present. Dorsal-edge view demonstrates invadopodia (red, arrows) inserted into the underlying gelatin (green). Ventral-edge view shows protrusive invadopodia and areas of gelatin degradation underneath the coverslip as regions of red present in the green matrix (arrowheads). The total image field presented is cropped to 77 x 65  $\mu$ m; the cell is ~ 60 x 40  $\mu$ m.

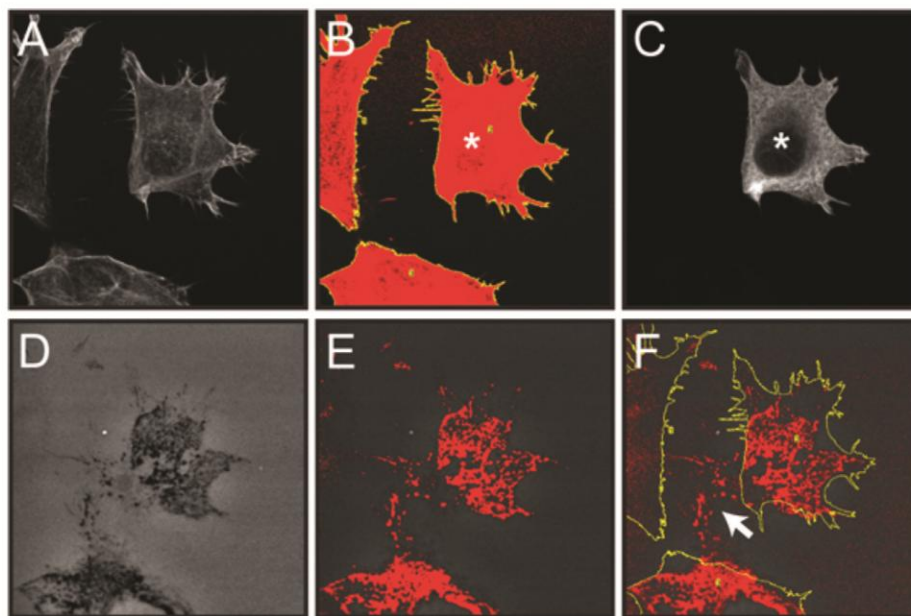
**Figure 5** shows some of the important steps for quantification of normalized gelatin matrix degradation as described in step 3 of the protocol. This procedure is designed to allow for unbiased quantitation of gelatin degradation in an entire field of view, and is suitable for matrix degradation attributed to many cells within the field.



**Figure 5.** Screen capture images demonstrating key steps in computational-assisted quantification of normalized gelatin degradation for cells within an entire microscopic image as described in protocol step 3. All fluorescent images have been converted to grayscale to better display the red thresholding and ROI markings. **A.** Image of Oregon Green 488-conjugated gelatin, showing dark areas ("holes") where degradation has occurred (step 3.4). **B.** Thresholded gelatin image highlighting areas of degradation in red (step 3.5). **C.** Drawing showing ROIs measured for area of degradation (step 3.7). **D.** Rhodamine phalloidin staining of F-actin (step 3.8). **E.** Thresholded actin image highlighting total cell area in red (step 3.9). **F.** Drawing showing cell areas to be measured (step 3.10). **G.** Image of DAPI-stained cell nuclei (step 3.13). **H.** Red outlines show results from automatic nuclei counting (step 3.13). The Watershed filter has the potential to separate nuclei that are touching (white arrow). If nuclei overlap extensively, they may not be separated into individual objects (red arrow). If a nucleus has an irregular shape, it may be separated into multiple objects (yellow arrow). **I.** Results from marking nuclei during a manual count using the cell counter tool (step 3.14).

**Figure 6.** demonstrates select steps involved in quantifying fluorescent gelatin degradation by individual cells within a mixed cellular population as described in protocol step 4. Here, matrix degradation by transfected cells can be analyzed within a mixed population of transfected and non-transfected cells.





**Figure 6.** Screen capture images of steps involved in quantifying gelatin degradation from individual transfected cells within a cell population. Quantification of a single transfected OSC19 cell overexpressing recombinant cortactin fused to the FLAG epitope tag is shown as an example. All fluorescent images have been converted to grayscale to better display the red thresholding and yellow ROI markings. **A.** Confocal image of three cells labeled with rhodamine-phalloidin (step 4.2). **B.** Drawing of total cell area based on F-actin staining following application of the Threshold and Analyze Particles functions (step 4.2-3). **C.** Confocal image of anti-FLAG immunolabeling of the cell population demonstrating a single cell expressing FLAG-tagged cortactin (marked with \*) (step 4.4). **D.** Image of Oregon Green 488-conjugated gelatin, showing dark areas ("holes") where degradation has occurred (step 4.5). **E.** Thresholded gelatin image highlighting dark areas of degradation in red (step 4.5). **F.** Thresholded gelatin image overlaid with cell outlines from panel B (step 4.6). Note that only the thresholded pixels within the cell outlines are counted in the analysis. Areas of degradation outside the current cell location (white arrow) result from cell migration across the gelatin over time and are not included in the analysis.

## Discussion

The ability to visualize cells degrading the extracellular matrix has aided in discovering the molecular mechanisms employed in the early steps of cell invasion. Pioneered by Wen-Tien Chen in the early 1980's<sup>4,14,15</sup>, coating fluorescently labeled extracellular proteins on glass coverslips for subsequent microscopic analysis has emerged as the primary technique in evaluating invadopodia function across a wide range of cell types. The prescribed protocol demonstrates the basic method used for preparing gelatin-coated coverslips that form a collagenous layer less than 2  $\mu\text{m}$  thick suitable for detection of extracellular matrix degradation by cells in most conventional fluorescent and confocal microscopes<sup>11,16-18</sup>, similar to what has been previously described<sup>19-21</sup>. These properties allow for rapid production of coated coverslips capable of detecting the initial onset of matrix degradation. The sensitivity afforded by the resulting thin gelatin matrix on the underlying hard glass surface likely aids in promoting invadopodia formation as a response to the high inherent stiffness of the overall matrix environment<sup>22</sup>. However, these matrices are not well suited for analysis of invadopodia elongation or additional morphological evaluation that has been achieved using thicker (30-100  $\mu\text{m}$ ) gelatin layers with similar methodology, coated transwells or electron microscopy<sup>20,23,24</sup>.

We have found that pre-conjugated commercially produced Oregon Green 488 gelatin allows for rapid experimental set up and consistent, reproducible results. However, alkaline borate conjugation of fluorescein isothiocyanate (FITC) to unlabeled gelatin remains a popular and inexpensive method for producing fluorescent gelatin conjugates<sup>20</sup>. Fibronectin is also used as an alternative matrix protein for labeling and coverslip coating<sup>4,9</sup>, and in some cases investigators have used labeled fibronectin layered onto unlabeled gelatin coated coverslips to create denser matrices<sup>11,25</sup>. Other matrices could be used, depending on the specifics of the cell type. In addition to dyes in the green 488 nm spectrum, a wide range of fluorophores have also been used with manual coupling methods to generate coverslips with different fluorescence spectra, including rhodamine<sup>21,26</sup>, Alexa Fluor 350<sup>24</sup>, 546<sup>21</sup>, 568<sup>5,11</sup>, 594<sup>27</sup> and 647<sup>5</sup> dyes. Such conjugates are easily adaptable for use in the prescribed protocol, providing the flexibility for utilizing specific ECM protein-dye combinations suitable for most any imaging filter set.

The techniques described herein provide the necessary detailed steps for utilizing ImageJ to quantify gelatin matrix degradation attributed to individual cells in a heterogeneous population or to entire cell groups as published previously<sup>6,28</sup>. Proprietary software has also been successfully employed for the same purpose<sup>5,25</sup>. In this protocol, the area of matrix degradation is normalized to either the total area of the cells or the total number of cells (nuclei) in the field. Generally, both options for normalization will give the same result (ELW, data not shown). However, if different cell lines having different sized cells are being compared or if the experimental treatment causes cells to change size, then it may be

more accurate to normalize to cell number. On the other hand, many cancer cell lines have a high percentage of multi-nucleated cells, in which case total cell area may be a more accurate parameter for normalization. Also, if only part of a cell is captured in an image (Figure 6), it may be better to normalize to cell area rather than underestimate the degradation potential for an individual cell. It is important to optimize the image analysis to best suit the characteristics and nuances of the specific experimental setup.

For determining cell numbers in a crowded field, counting nuclei is often the method of choice. ImageJ has a nucleus counter plugin for automatic counting. One option in this tool is the Watershed filter. This filter will help separate nuclei that are touching by segregating them into individual objects (Figure 5H, white arrow). However, this filter may not be able to separate nuclei that overlap extensively (Figure 5H, red arrow). In addition, if a nucleus has an irregular shape and large variations in intensity, the filter may separate a single nucleus into multiple objects (Figure 5H, yellow arrow). Therefore, it is important to try different thresholding and smoothing methods in this plugin to determine the best parameters for analysis. If the automatic counting does not produce accurate numbers, the cell counter plugin can facilitate manual counting of cells or nuclei.

In cases utilizing transient transfection, images will often contain a mixture of cells expressing or not expressing a protein of interest (Figure 6). In this scenario, it is not always apparent which cells were responsible for creating areas of matrix degradation. This is especially true if the cells are migrating across the gelatin. To be consistent in the analysis, it is important to only measure the degraded areas directly underneath each cell. By thresholding to select the dark areas in the matrix and using the actin to generate cell outlines, only the degraded areas under the cells will be quantitated. This procedure will exclude degraded areas outside of cell boundaries from analysis (Figure 6F, arrow). The assay may require optimization to select a time point that allows sufficient time for degradation before the cells have had a chance to move.

Numerous methods have been developed to quantitate invadopodia formation and function. In addition to matrix degradation, other frequently reported parameters include determining the number of invadopodia per cell, the percentage of cells displaying invadopodia within a given population, and the number of "immature" or "pre" non-degrading invadopodia compared to "mature" invadopodia capable of degrading the ECM<sup>11,13,25,26</sup>. The method(s) of choice for invadopodia evaluation depend on inherent characteristics of each cell type. For instance, counting the number of invadopodia per cell or determining the percentage of cells containing invadopodia is a straightforward approach that works well if the analyzed cells contain just a few prominent invadopodia, but becomes more difficult in cells that have dozens of invadopodia or where invadopodia may be small and difficult to detect. Using the degradation assay makes it possible to calculate the percentage of pre-invadopodia vs. mature invadopodia in single cells or in a population by comparing the total number of cells with invadopodia to the percentage that are degrading matrix. If there is a discrepancy where fewer cells are degrading matrix compared to cells displaying invadopodia, it may indicate that these cells are forming pre-invadopodia that were incapable of matrix degradation at the time the cells were fixed.

Whatever method combination is chosen for analysis, it is important to quantify the desired invadopodia characteristics as objectively as possible. When collecting images on the microscope, choose fields by looking at cells (actin), rather than the fluorescent matrix, to avoid bias from preferentially selecting areas with high levels of degradation. Multiple images should be acquired to ensure a fair representation of the cell population. Images should also be acquired at an appropriate magnification. For uniform populations of cells, lower magnification can be used to collect more cells as long as the areas of degradation can still be resolved. Higher magnification images are preferred to measure areas under individual cells and to resolve individual invadopodia. When areas are being quantitated, thresholding images based on intensity is more objective than manually choosing the area of the matrix to measure. In all cases, a sufficient number of cells from multiple independent experiments should be analyzed to give statistically meaningful, reproducible results.

## Disclosures

No conflicts of interest declared.

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Materials List for:

## Quantitative Measurement of Invadopodia-mediated Extracellular Matrix Proteolysis in Single and Multicellular Contexts

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URL: <http://www.jove.com/video/4119/>

### Materials

| Name                                | Company                      | Catalog Number | Comments  |
|-------------------------------------|------------------------------|----------------|---|
| gelatin                             | Sigma                        | G1890          | Porcine skin  |
| sucrose                             | Fisher                       | BP220          |   |
| 12 mm coverslips                    | Fisher                       | 50-121-5159    |   |
| 24 well plates                      | Fisher                       | 08-772-1       | BD Falcon   |
| nitric acid                         | Ricca                        | R5326000       | 20% solution  |
| poly-L-lysine                       | Electron Microscopy Sciences | 19320-B        | 0.1% solution   |
| glutaraldehyde                      | Sigma                        | G7526          | 8% solution   |
| Oregon Green 488-conjugated gelatin | Invitrogen                   | G-13186        |   |
| sodium borohydride                  | Sigma                        | 213462         |   |
| Triton X-100                        | Fisher                       | BP151          |   |
| rhodamine-phalloidin                | Invitrogen                   | R415           |   |
| anti-cortactin (clone 4F11)         | Millipore                    | 05-180         |   |
| Anti-FLAG antibody                  | Millipore                    | MAB3118        |   |
| Alexa Fluor 647 Goat anti-mouse IgG | Invitrogen                   | A21235         |   |
| ProLong Gold antifade               | Invitrogen                   | P36930         |   |
| LSM 510 Confocal Microscope         | Zeiss                        |                |   |
| ImageJ software                     | Public domain                |                | <a href="http://www.macbiophotonics.ca/imagej/">http://www.macbiophotonics.ca/imagej/</a> |
| AutoQuant X2.2 software             | Media Cybernetics            |                |   |
| NIS Elements software               | Nikon                        |                |   |



Video Article

# Multi-photon Imaging of Tumor Cell Invasion in an Orthotopic Mouse Model of Oral Squamous Cell Carcinoma

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URL: <http://www.jove.com/video/2941/>

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Keywords: Medicine, Issue 53, Invasion, mouse model, two-photon microscopy, tongue, orthotopic, head and neck cancer

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## Abstract

Loco-regional invasion of head and neck cancer is linked to metastatic risk and presents a difficult challenge in designing and implementing patient management strategies. Orthotopic mouse models of oral cancer have been developed to facilitate the study of factors that impact invasion and serve as model system for evaluating anti-tumor therapeutics. In these systems, visualization of disseminated tumor cells within oral cavity tissues has typically been conducted by either conventional histology or with *in vivo* bioluminescent methods. A primary drawback of these techniques is the inherent inability to accurately visualize and quantify early tumor cell invasion arising from the primary site in three dimensions. Here we describe a protocol that combines an established model for squamous cell carcinoma of the tongue (SCOT) with two-photon imaging to allow multi-vectorial visualization of lingual tumor spread. The OSC-19 head and neck tumor cell line was stably engineered to express the F-actin binding peptide LifeAct fused to the mCherry fluorescent protein (LifeAct-mCherry). Fox1<sup>nu/nu</sup> mice injected with these cells reliably form tumors that allow the tongue to be visualized by *ex-vivo* application of two-photon microscopy. This technique allows for the orthotopic visualization of the tumor mass and locally invading cells in excised tongues without disruption of the regional tumor microenvironment. In addition, this system allows for the quantification of tumor cell invasion by calculating distances that invaded cells move from the primary tumor site. Overall this procedure provides an enhanced model system for analyzing factors that contribute to SCOT invasion and therapeutic treatments tailored to prevent local invasion and distant metastatic spread. This method also has the potential to be ultimately combined with other imaging modalities in an *in vivo* setting.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/2941/>

## Protocol

### 1. Cell Lines, Vector Construction and Lentiviral Production

1. Human head and neck tumor cells lines (OSC19 or UMSCC1) were cultured in complete media consisting of DMEM (Cellgro cat # 50-003-PB) supplemented with 10% fetal bovine serum FBS (Hyclone cat # SH30070.03), 1% penicillin/streptomycin (Cellgro cat #30-002-CI), and 1% non-essential amino acids (Cellgro cat # 25-025-CI).
2. To transfer the LifeAct-mCherry coding sequence into the pLL7.0 lentiviral vector, the Sbf1 recognition site in the parent mCherry cDNA was altered by introducing three silent mutations into the recognition sequence using site-directed mutagenesis (Stratagene cat # 200518-5). The resulting modified LifeAct-mCherry sequence was then PCR amplified with flanking EcoR1/Sbf1 sites and subcloned into pLL7.0 to generate the pLL7.0-LifeAct-mCherry construct.

### 2. pLL7.0-LifeAct-mCherry Virus Production

1. Viral production was conducted according to the Lentiviral Expression Systems manual (System Bioscience version 2-051018).
2. The packaging cell line 293T/17 cells (ATCC cat # CRL-11268) was grown to 40% confluence in the same complete media used for HNSCC lines.
3. Cells were transfected with the pLL7.0-LifeAct-mCherry, psPAX2, and pVSV-G vectors in a 3:2:1 ratio, respectively using CalPhos (Clontech cat # 631312).
4. After 24 hours, the initial media from the transfection was replaced with fresh media.
5. Media was then collected and replenished every 12 hours for 72 hours and stored at 4°C.

### 3. Production of Head and Neck Cell Lines with Stable LifeAct-mCherry Expression

1. The collected media was spun at 2000 rpm for 10 minutes at 4°C.
2. One ml of clarified media containing virus was directly added to OSC19 or UMSCC1 cells for 12 hours. Cells were then rinsed, and an additional one mL of virus was added for another 12 hour period.
3. Cells were treated with media containing 200mg/ml puromycin for two weeks to select resistant colonies.
4. Surviving clones were screened visually for LifeAct-mCherry expression by fluorescence microscopy. Individual positive colonies were trypsinized using sterile 3mm cloning discs (Fisher cat # 0790710A).
5. Positive cells were maintained in media containing 200mg/ml puromycin until frozen back or used for orthotopic injection.

### 4. Orthotopic Tumor Xenograft Formation

1. All animal procedures were conducted in accordance with a protocol (09-0821) approved by the West Virginia University Animal Care and Use Committee.
2. LifeAct-mCherry expressing tumor cells were trypsinized, centrifuged and  $2.5 \times 10^4$  cells were resuspended in 50  $\mu$ L complete media.
3. Tumor cells were loaded into a one ml syringe attached to a 27  $\frac{1}{2}$  gauge needle.
4. Female athymic Fox1<sup>nu/nu</sup> mice 8 weeks of age (Harlan Laboratories) were anesthetized with combination of 80mg/kg ketamine and 10mg/kg xylazine. Anesthetized mice were maintained between 37-40 °C on a heating pad.
5. Using sterile forceps, the tip of the tongue was gently grasped and carefully pulled out of the oral cavity.
6. Cells were slowly injected into one side of each tongue to create a bulbous mass in the tongue center, avoiding the lingual arteries.
7. Mice were injected with 2.1mg/kg yohimbine and returned to the heating pad where they were monitored for 2-3 hours during recovery from anesthesia.
8. Once revived, mice were placed into sterile cages containing a soft transgenic dough diet (Bioserve cat # S3472).
9. Mice were weighed every 2-3 days and monitored visually for tumor onset.

### 5. Preparation of Mouse Tongues for *Ex-vivo* Imaging

1. Mice harboring tumors at different time points (typically 2-4 weeks post-injection) were euthanized by carbon dioxide inhalation.
2. Tongues were extracted, rinsed with 1X PBS and attached to one side of a conventional paraffin tissue embedding cassette (StatLab Cat #H104,) using monofilament sewing thread from a local hobby shop and a size 8 sewing needle.
3. Once the tongues were immobilized, the entire cassette assembly was placed in a 30mm tissue culture dish and immersed in 1X PBS.
4. Processed tongues were immediately used for two-photon excitation microscopy.

### 6. Imaging of Tongue Tumors with Two-photon Microscopy

1. Tongue cassettes were submerged in a 60mm dish containing 1X PBS secured in a custom designed holder on a retractable cantilever arm (Chamber Shuttle, Siskiyou Instruments) positioned under the objective of an upright microscope (Moveable Microscope (MOM); Sutter Instruments).
2. A 40X/0.8NA water dipping objective lens was placed directly on or over visible tumor lesions. Tongues were imaged by two-photon microscopy with the Ti:sapphire laser (Mira, Coherent) intensity at 60 mW and input wavelength of 755 nm to optimize the mCherry signal.
3. Serial 1mm laser scanning images were collected at 1  $\mu$ m incremental depths over a total tissue depth between 15 and 100  $\mu$ m (depending on tumor volume). Images were captured using ScanImage, an open source program based on the MATLAB platform that was developed by the Karel Svoboda laboratory (Janelia Farms, HHMI). ScanImage generates a two-channel output of raster scan patterns to control the x/y galvanometric scan mirrors, and at the same time captures a maximum four-channel signal input simultaneously from photomultiplier tubes (PMTs) through a data acquisition board (PCI-6610S, National Instruments). The PMT signals are amplified by low noise current preamplifiers (SR570, Stanford Research System) before feeding into the NI-DAQ board for display on the monitor screen. ScanImage collects z-stack images by controlling the z-axis of the objective and collects time-lapse images in a single or cycle mode. Images were saved in a single TIFF file with 16 bit depth.

### 7. Image Analysis using Amira Software

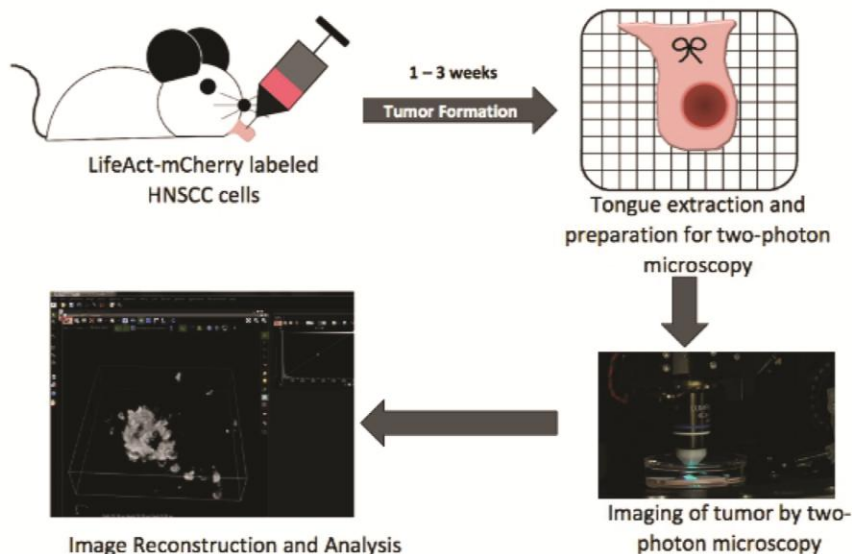
1. Amira imaging for tumor quantification: In Amira software, open the TIFF file containing the set of z-stack images.
2. Highlight the file name, select and apply the Voltex function to generate a three dimensional rendering. A large primary tumor image with several smaller, dissociated invasive groups (IG) of collectively invaded cells is typically apparent in the rendered image (see Figure 6A).
3. To select the primary tumor mass, select "Open Data", then "Labeling", then "Label Field".
4. Thresholding the primary tumor- select all z-stack images and scroll through the images in the z-plane to ensure inclusion of only the primary tumor mass within three dimensions. This is typically the largest sized image in the field and often appears segmented as the imaged is traversed through the z-plane.
5. Use the magic wand/threshold function to correct background fluorescence and eliminate it from the image without discarding any of the tumor signal. Highlight the "Inside File" label and click the # button. Select the "All Slices" check box. This produces a colored border around the thresholded primary tumor area in every z-stack increment.
6. To select IGs, choose the "New" function. Select a single IG and ensure it is not associated with the primary tumor by scrolling through the z-plane.
7. Select "All Slices" and click the # button. Rename the file (ex: IG 1)
8. Repeat the thresholding procedure in 7.4 and the highlighting step in 7.5 for the determined IG. Select an outline color different than the color used to denote the primary tumor mass.

9. Repeat as necessary for each additional IG throughout the image, scanning through the z-plane to ensure all IGs are denoted. Using different colors for each identified IG aids in future identification on the image.
10. Once the primary tumor and all IGs are selected, select "Segmentation" from the pulldown menu, then select "Material Statistics". This provides the volume measurement as well as the X, Y and X tumor core coordinates for the primary tumor in order to calculate distances of IGs from the central tumor point.
11. With the volumes calculated, determine the distance for each IG from the central core of the primary tumor. Select "Segmentation", then "Material Statistics". This step calculates all measurements into pixels. Convert pixels to micrometers based on the calibration of the microscope objective and any additional increases in magnification (i.e.; zoom functions). For the microscope and settings used in these experiments, the 40X objective was used with no zoom, giving a calibration of 1 pixel = 0.298  $\mu\text{m}$ . Import data into an Excel spreadsheet, which gives parameters for the primary tumor in three dimensions (X1, Y1, Z1) and for each IG (ex: X2, Y2, Z2 for the first IG).
12. The invaded distance in microns for each IG from the center of the primary tumor is calculated using the formula  $\sqrt{(X2-X1)^2 + (Y2-Y1)^2 + (Z2-Z1)^2}$ .
13. The tumor invasive index ( $T_I$ ) is calculated using the formula  $T_I = N_T \times V_T \times D_T$ , where  $N_T$  = the total number of IGs in the image,  $V_T$  = the total volume of all IGs,  $D_T$  = total distance traveled of all invasive groups from the center of the primary tumor.

## 8. Three dimensional renderings with Nikon NIS-Elements Software

1. Three dimensional renderings with greater topographical detail can be generated by importing the original 16-bit monochrome TIFF files of the entire tumor image into the Nikon NIS-Elements software package (Nikon, Melville, NY). Select "File", then "ND", then "Create ND File from File Sequence". Select the TIFF z-series image stack and specify the appropriate step size.
2. Calibrate the ND document in the xy plane. Specify the size of one pixel using a manual calibration.
3. Choose the "Volume View". Use the "HQ" function to calculate additional slices in the z-plane for higher quality. In the "3D Renderer Settings", use the "Advanced Renderer" with a quality of "Ultra High Details" and "Full Resolution". Choose "Alpha Blending" to accentuate the tumor surfaces.
4. Optimize the three-dimensional image for presentation. Zoom in on the tumor and associated IGs and crop as needed. Rotate the image in the xyz planes and adjust the LUTs.
5. Capture the image for presentation. Select "Edit- Create View Snapshot (8 bit RGB)". Name and save file accordingly.

## 9. Representative Results

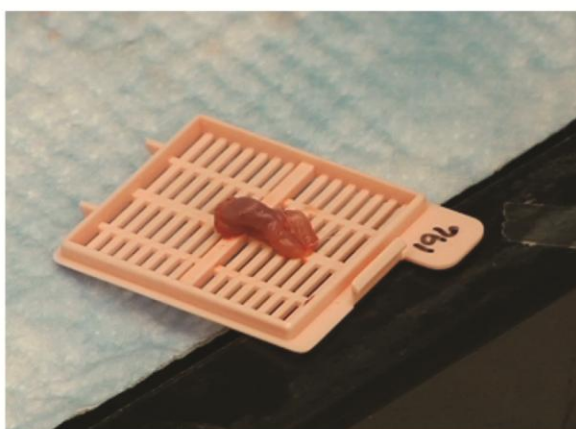


**Figure 1.** Overall schematic illustrating key steps in orthotopic tongue tumor production and in situ two photon imaging.

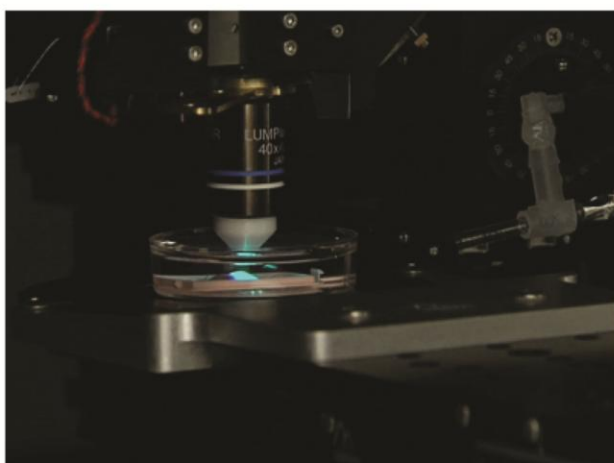




**Figure 2.** Injection of LifeAct-mCherry expressing OSC19 cells into the mouse tongue.



**Figure 3** Resected tumor-containing mouse tongue prepared for two photon imaging.



**Figure 4.** Orientation of a tumor-containing tongue in position on a two photon microscopy ready for imaging.

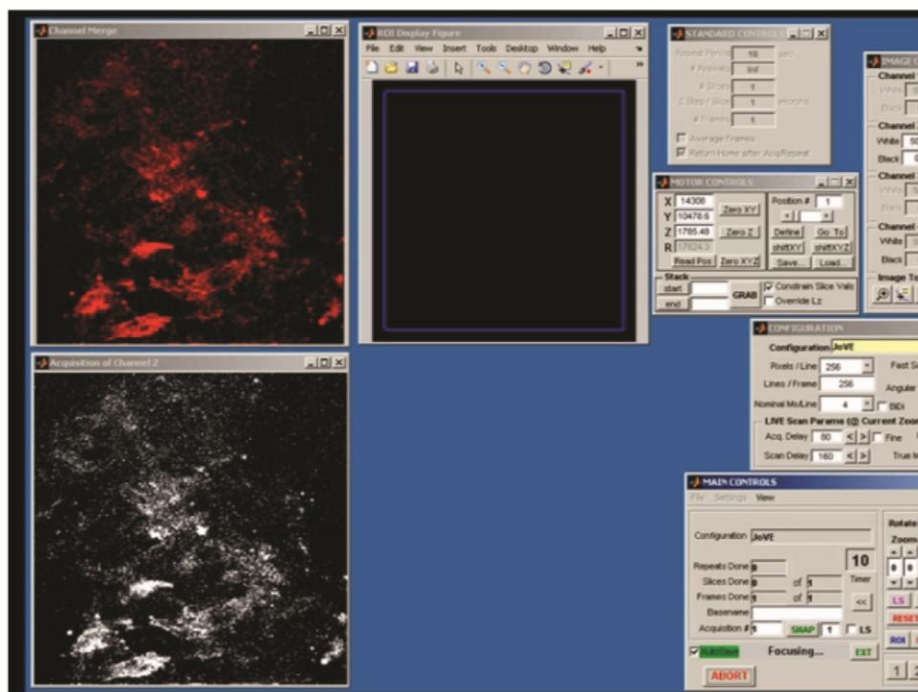
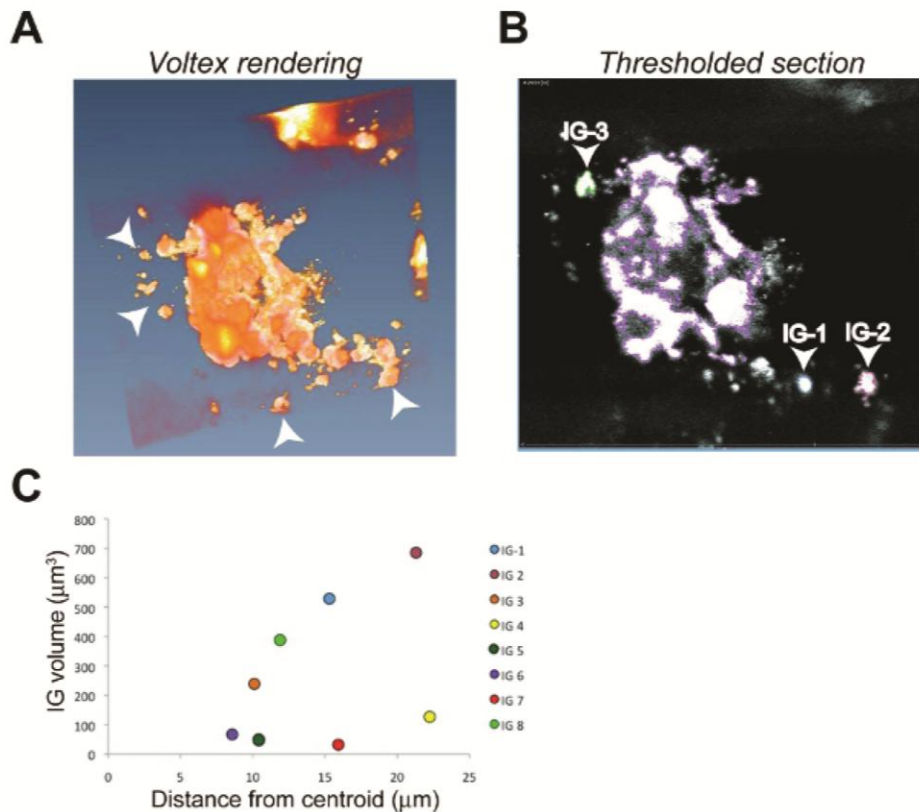
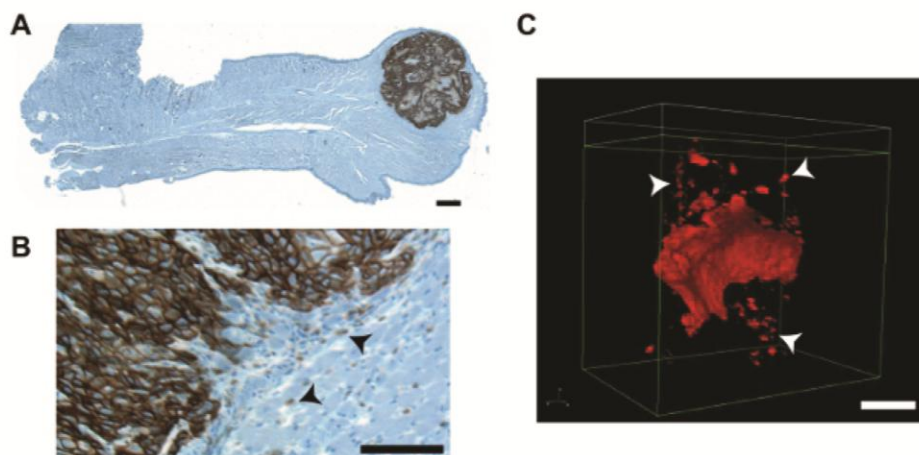


Figure 5. Representative screen shot from ScanImage demonstrating raw data acquisition of the initial two-photon image.



**Figure 6.** Image analysis and quantification of tumor invasion in an orthotopic OSC19 tumor. Representative screen shot images of Amira Voltex rendering (**A**) and a single thresholded z-section (**B**) with the primary primary tumor outlined in purple, invasive group 1 (IG-1) outlined in blue, IG-2 outlined in red and IG-3 outlined in green as an example of the identification procedure. Arrowheads denote IGs in (**A**) and (**B**). **C.** Plots of volume versus invaded distance for eight individual IGs used to calculate the tumor invasion index ( $T_i$ ).



**Figure 7.** Representative images of tumors and invaded tumor groups from this protocol compared with images from a conventional IHC approach. **A.** Paraffin section of an entire mouse tongue harboring a UMSSC1 orthotopic tumor. Immunohistochemical staining was conducted using conventional procedures with a primary antibody against the HNSCC-specific cell marker emmprin (Zymed; cat # 34-5600) at 1:1000 dilution and visualized by using the OmniMap DAB anti-Rb detection kit (Ventana cat # 760-149) followed by iron hematoxylin staining. Images encompassing the total tongue area were collected individually at 4X magnification on an Olympus ZX70 Provis microscope with an Optronics MicroFire CCD camera and reconstructed using the StereoInvestigator imaging package (MBF Bioscience). The tumor is evident at the tongue

tip. An enlarged region showing the invasive front and individual tumor cell groups (arrowheads) is shown in (B). C. Nikon NIS-Elements rendering of a representative OSC19 tumor. Enhanced contour detail and clear visualization of IGs (arrowheads) is evident. Bars in all images = 100  $\mu$ m.

## Discussion

Orthotopic mouse models have proven useful for studying many aspects of head and neck cancer<sup>1,2</sup>. We have combined a well established orthotopic system of SCOT<sup>3</sup> with two-photon microscopy imaging of mCherry-labeled cells as a system to study the early events of head and neck tumor cell invasion. In this procedure, we have noted that cells can leak from the site of tumor injection, especially in mice six weeks or younger due to insufficient tongue size. We use older mice to avoid this issue. The larger tongue size with older mice also aids in avoiding rupture of a lingual artery and excessive hemorrhaging from the tongue. Tumor take is greatly enhanced when the tongue dramatically swells at the site of initial injection. This swelling subsides within one-two hours as the injected fluid is absorbed. Tumor growth appears evident one-two weeks post injection with the indicated injected cell number as a small white bump on the tongue surface. We also note that at the 40X objective used in our studies presented here, we cannot distinguish individual tumor cells but do identify IGs as invasive cell clusters that recapitulate the typical mode of HNSCC invasion.

To date this model has been extensively used for testing the role of specific molecules as well as several anti-tumor drugs on SCOT growth an invasion, with efficacy measured by cervical lymph node metastasis monitored using IHC or bioluminescent methods<sup>4-7</sup>. Tumors formed in this system manifest close to the tongue surface (Figure 7), allowing the application of two-photon microscopy to image entire tumors in situ as invasive multi-cellular clusters. The procedure can also be utilized to visualize tumor invasion with cellular resolution. Two-photon microscopy has been previously utilized to study experimental treatments for head and neck cancer in orthotopic<sup>8,9</sup> and xenograft<sup>8,10</sup> models. However, there are two major differences between these reports and our protocol. First, these studies use extracellular labels to target/identify head and neck tumor cells, possibly limiting detection only to tumor cells with ample access to the circulation. Second, invasion of tumor cells close to the primary site that likely recapitulates early metastatic activity was not assessed as an experimental parameter. Our protocol provides the ability to directly quantify tumor cell invasion at any point during tumor progression in mouse tongues. While the method described here describes the procedure using dissected tongues, we are currently in the process of adapting this method to image tumor invasion in live mice for use in combination with bioluminescence to simultaneously monitor early invasion, local lymph node involvement and distant metastasis in the same animal. Alterations to the protocol for in vivo imaging require designing of an appropriate stage for positioning and maintaining mice during imaging, as well as a practical system to properly irrigate the oral cavity of anesthetized mice during the imaging procedure. Once optimized, these adaptations will provide the ability to study the role of potential pro-invasive molecules and the testing anti-invasive compounds on local invasion and more distant metastatic involvement in animals over extended time periods.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

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## Revisiting the ERK/Src cortactin switch

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**T**he filamentous (F)-actin regulatory protein cortactin plays an important role in tumor cell movement and invasion by promoting and stabilizing actin related protein (Arp)2/3-mediated actin networks necessary for plasma membrane protrusion. Cortactin is a substrate for ERK1/2 and Src family kinases, with previous *in vitro* findings demonstrating ERK1/2 phosphorylation of cortactin as a positive and Src phosphorylation as a negative regulatory event in promoting Arp2/3 activation through neuronal Wiskott Aldrich Syndrome protein (N-WASp). Evidence for this regulatory cortactin “switch” in cells has been hampered due to the lack of phosphorylation-specific antibodies that recognize ERK1/2-phosphorylated cortactin. Our findings with phosphorylation-specific antibodies against these ERK1/2 sites (pS405 and pS418) indicate that cortactin can be co-phosphorylated at 405/418 and tyrosine residues targeted by Src family tyrosine kinases. These results indicate that the ERK/Src cortactin switch is not the sole mechanism by which ERK1/2 and tyrosine phosphorylation events regulate cortactin function in cell systems.

Motility-based processes in normal and transformed cells are governed by signal transduction pathways that regulate actin cytoskeletal dynamics. Actin regulatory proteins that serve as substrates downstream of multiple kinase cascades are important intersection points in integrating and controlling motile and invasive activities. Cortactin is an F-actin binding adaptor protein initially identified as a Src substrate in v-Src transformed cells.<sup>1,2</sup>

Subsequent work identified three tyrosine residues within the cortactin proline-rich carboxyl-terminal domain (Y421, Y470 and Y486 in humans) that are phosphorylated by Src and other tyrosine kinases.<sup>3,4</sup> Analysis of these tyrosine residues using phenylalanine point mutants indicates the importance of their phosphorylation in cell motility and tumor cell metastasis.<sup>3,5</sup> Tyrosine phosphorylated cortactin localizes within lamellipodia of motile cells and invadopodia in invasive carcinoma cells,<sup>6,7</sup> supporting a functional role in cell movement and invasion.

Cortactin is a substrate for multiple serine/threonine kinases in addition to serving as a tyrosine kinase substrate.<sup>8</sup> ERK1/2 is a prominent serine/threonine kinase that phosphorylates cortactin at serine 405 and 418 in response to epidermal growth factor receptor (EGFR) activation.<sup>9</sup> Cortactin contains a Src homology (SH)3 domain at its carboxyl terminal end that is capable of binding and activating N-WASp, resulting in enhanced Arp2/3 actin nucleation activity.<sup>10,11</sup> In 2004, Martinez-Quiles et al. examined the functional impact of ERK1/2 and Src phosphorylation on the ability of cortactin to regulate N-WASp activity. This report utilized purified protein components in *in vitro* actin polymerization assays to demonstrate that ERK1/2 phosphorylation substantially enhances the ability of cortactin to bind and activate N-WASp, promoting Arp2/3 actin nucleation. In contrast, Src phosphorylation of cortactin prevents cortactin binding to N-WASp and ablates the ability of phosphomimetic 405/418 cortactin mutants to promote N-WASp activation. These results led to the proposal of an on-off switch mechanism for

**Key words:** cortactin, ERK1/2, Src, N-WASp, phosphorylation, motility, invasion

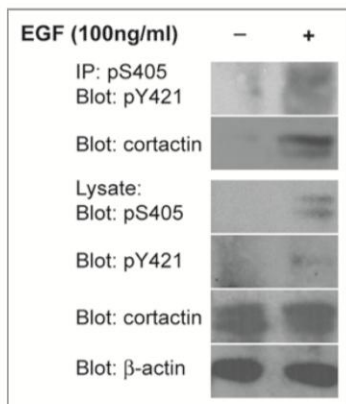
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**Figure 1.** Cortactin is co-phosphorylated on S405 and Y421. OSC19 oral squamous carcinoma cells serum starved for 24 h were stimulated with 100 ng/ml EGF for 20 min as indicated. Cell lysates were immunoprecipitated with anti-cortactin pS405 antibodies and analyzed by western blotting with anti-cortactin pY421 and pan-cortactin antibodies. Total cell lysates were evaluated for the presence of pS405 cortactin, pY421 cortactin, total cortactin and  $\beta$ -actin.

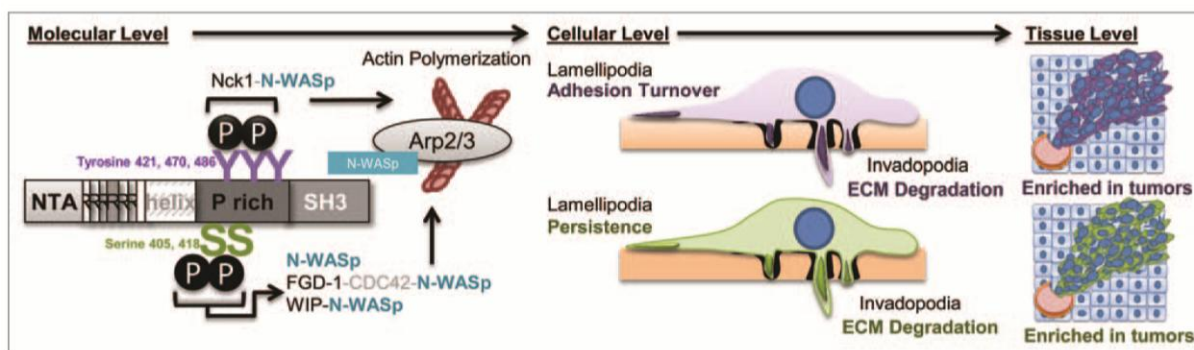
cortactin regulation of N-WASp activity, whereby ERK1/2 cortactin phosphorylation liberates the cortactin SH3 domain from an undefined autoinhibitory state (presumed to be a SH3 domain binding site within the proline-rich domain), allowing it to bind and activate N-WASp. Src phosphorylation terminates this activity, enabling cortactin to return to its inactive conformation. Based on this model, ERK1/2 phosphorylation and Src tyrosine

phosphorylation are functionally interdependent events.

Subsequent studies utilizing exogenously expressed phosphomimetic and phosphorylation incompetent S405/418 mutants supports a role for ERK1/2 cortactin phosphorylation in promoting intracellular actin polymerization<sup>12</sup> and actin-dependent invadopodia matrix degradation activity.<sup>13</sup> Our development of site-specific antibodies against cortactin pS405 and pS418 allows for the first direct cellular evaluation of these phosphorylation sites. We demonstrate that pS418 cortactin localizes to lamellipodia and invadopodia in carcinoma cells, supporting the identified role for cortactin in actin polymerization derived from studies with point mutant constructs. Significantly, these antibodies, along with commercially available antibodies against cortactin pY421 (and likely anti-pY470/pY486 or equivalent antibodies), provide a means for directly assessing cortactin serine and tyrosine phosphorylation status derived from cellular preparations. Cortactin is phosphorylated on S405/418 and Y421 in lysates from EGF-stimulated UMSSC2 head and neck squamous carcinoma cells, indicating that ERK1/2 and Src (or other cortactin-targeting tyrosine kinases) are activated and phosphorylate cortactin following EGFR activation. Analysis of phosphorylation-null point mutants with anti-pY421 and pS405 antibodies indicates that ERK1/2 and cortactin tyrosine phosphorylation are not interdependent

events, since the inability of cortactin to become tyrosine phosphorylated does not prevent ERK1/2 phosphorylation (and vice versa). Furthermore, cortactin immunoprecipitated with anti-pS405 antibodies is phosphorylated on pY421 (Fig. 1), demonstrating co-phosphorylation of these sites on the same cortactin molecule. These data indicate that at least a subpopulation of cortactin within tumor cells is simultaneously phosphorylated by ERK1/2 and tyrosine kinases.

Our findings provide evidence that the ERK/Src cortactin switch is not the primary phospho-regulatory cortactin mechanism employed by cells. Recent studies indicate that cortactin tyrosine phosphorylation promotes actin polymerization through recruitment of the adaptor NCK1, which in turn binds and activates N-WASp and Arp2/3 to stimulate actin network formation in invadopodia.<sup>7,14,15</sup> The concurrent ability of cortactin to activate N-WASp by both tyrosine and ERK1 phosphorylation events allows for amplification of Arp2/3-mediated actin polymerization based on specific signaling input, providing a means for fine-tuning actin regulation at dynamic membrane structures during migration and invasion. Cortactin regulation of actin networks is likely more complex, given the ability of the cortactin SH3 domain to interact and activate several proteins in addition to N-WASp that signal to control Arp2/3 activation or actin dynamics.<sup>16-20</sup> The presence of tyrosine<sup>21</sup> and



**Figure 2.** Mechanistic and cellular roles of cortactin tyrosine and serine phosphorylation in tumor cell motility. Tyrosine (purple) and serine (green) phosphorylation events are shown depicting direct and indirect pathways that ultimately result in N-WASp activation. The effects of cortactin serine and tyrosine phosphorylation result in common net outcomes in invadopodia function (ECM degradation), but differ in impact on lamellipodia dynamics as shown. Cortactin phosphorylated on serine and tyrosine residues is enriched in human tumors, where it likely enhances invasive and metastatic capacity.

serine phosphorylated cortactin in head and neck squamous cell carcinoma tumors suggests that both tyrosine- and serine-based signaling is relevant in neoplastic progression.

In addition to the apparent redundant roles of tyrosine and serine phosphorylation in N-WASP activation, these cortactin phosphorylation events can also have divergent cellular functions in migration. In two-dimensional systems, cortactin tyrosine phosphorylation alters focal adhesion turnover, whereas serine 405/418 phosphorylation stimulates actin polymerization and motility.<sup>12</sup> Our work extends these findings by demonstrating that serine 405/418 phosphorylation is required for dominant lamellipodia persistence, whereas tyrosine phosphorylation has no effect on lamellipodia dynamics (Ammer and Weed, unpublished data). This suggests that there are context-specific roles for cortactin tyrosine and serine phosphorylation in tumor cell motility (Fig. 2) and is in agreement with the ability of these phosphorylation events to regulate different aspects of endocytic membrane trafficking.<sup>22-24</sup> Continued deciphering of the complex pathways that impinge on and emanate from serine- and tyrosine-phosphorylated cortactin continues to present interesting and challenging avenues for understanding how these signals are utilized and integrated during different phases of cancer cell motility.

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## Further insights into cortactin conformational regulation

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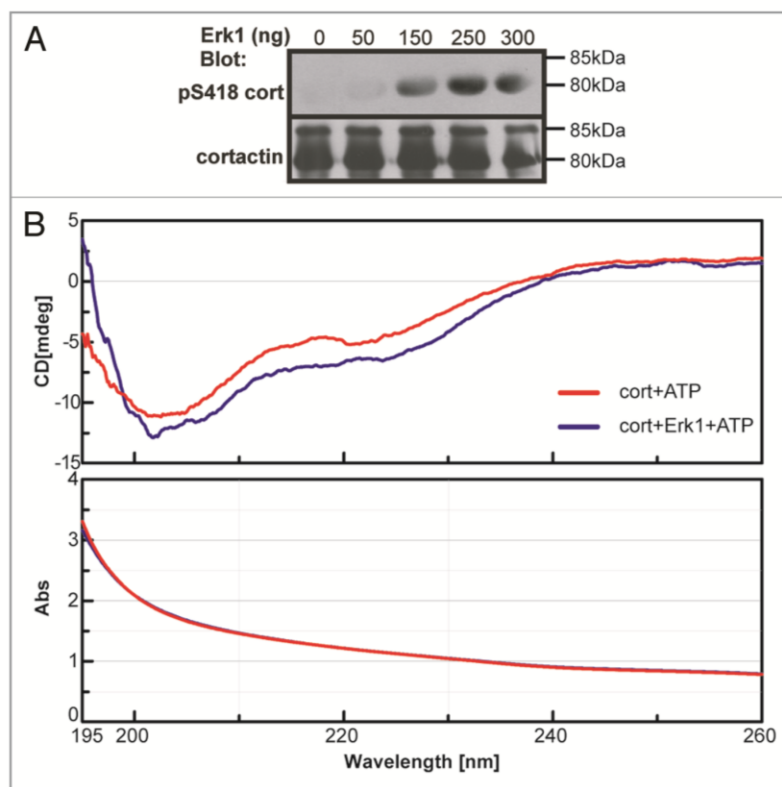
The actin regulatory protein cortactin is involved in multiple signaling pathways impinging on the cortical actin cytoskeleton. Cortactin is phosphorylated by ERK1/2 and Src family tyrosine kinases, resulting in neuronal Wiskott Aldrich Syndrome protein (N-WASp) activation and enhanced actin related protein (Arp)2/3-mediated actin nucleation. Cortactin migrates as an 80/85 kDa doublet when analyzed by SDS-PAGE. Phosphorylation by ERK1/2 is associated with conversion of the 80 kDa to the 85 kDa form, postulated to occur by inducing a conformational alteration that releases the carboxyl-terminal SH3 domain from autoinhibition. Our recent analysis of the 80–85 kDa cortactin “shift” in tumor cells indicates that while ERK1/2 phosphorylation is associated with the 85 kDa shift, this phosphorylation event is not required for the shift to occur, nor does ERK1/2 phosphorylation appreciably alter global cortactin conformation. These data indicate that additional factors besides ERK1/2 phosphorylation contribute to generating and/or maintaining the activated 85 kDa cortactin form in stimulated cells.

Changes in protein conformation are important for generating and propagating intracellular signal transduction events. During normal and pathogenic cell motility, regulation of actin cytoskeletal dynamics responsible for generating movement is dependent on conformational alterations in protein relay systems that activate and terminate signaling pathways responsible for initiating and maintaining motility. Proteins at the intersection of this molecular circuitry are key mediators

in motility-driven signal regulation. The filamentous (F)-actin binding protein cortactin interacts with Arp2/3 complex to stimulate and stabilize Arp2/3-F-actin networks in lamellipodia and invadopodia of motile and invasive tumor cells.<sup>1,2</sup> Cortactin is also a substrate for multiple protein kinases; most notably by ERK1/2 at S405 and S418,<sup>3</sup> and Src family tyrosine kinases at Y421, Y470 and Y486.<sup>2</sup> The ERK1/2 and Src phosphorylation sites all reside within a proline-rich region adjacent to a SH3 domain at the extreme carboxyl terminus.<sup>2,3</sup> Phosphorylation of cortactin by ERK1/2 and tyrosine kinases promotes tumor cell migration and is required for invadopodia-mediated extracellular matrix degradation activity,<sup>4,5</sup> demonstrating important functional roles for these phosphorylation events in cancer progression.

At the mechanistic level, cortactin tyrosine phosphorylation stimulates pro-invasive activity by providing binding sites at Y421 and Y466 for the SH2 domain of the adaptor protein NCK1.<sup>6–8</sup> NCK1, through its SH3 domain, binds N-WASp to release an acidic carboxyl-terminal (VCA) domain that is responsible for binding to and activating Arp2/3 complex, promoting actin polymerization.<sup>9,10</sup> In the case of ERK1/2, cortactin phosphorylation at S405/418 promotes association of the carboxyl-terminal SH3 domain with N-WASp, resulting in a similar Arp2/3 activation cascade.<sup>11</sup>

Cortactin purified from most cell types as well as from recombinant cell-free sources typically migrates as an 80/85 kDa doublet in SDS-PAGE gels.<sup>12–14</sup> This doublet most likely represents a single polypeptide, since electrophoresis in urea-containing



**Figure 1.** Analysis of cortactin secondary structure following ERK1 phosphorylation. (A) Phosphorylation of cortactin by ERK1. Recombinant cortactin (25  $\mu$ g) was phosphorylated with the indicated amounts of ERK1 at 30°C for 30 min. Aliquots of each reaction were analyzed by western blotting with anti-pS418 and total cortactin antibodies. The positions of the 80 kDa and 85 kDa forms are denoted. (B) Kinase reactions containing recombinant cortactin (25  $\mu$ g) and ATP (500  $\mu$ M) incubated without (red line) or with (blue line) 250 ng ERK1 were analyzed with a Jasco J-810 Spectropolarimeter using a scan speed of 50 nm/min with a 4 sec response time, 1 nm band width and 0.1 nm data pitch (top). The absorbance for each reaction was monitored throughout the analyzed wavelength range (bottom) to control for sample-to-sample variability. Scans are shown for each reaction condition following rendering with Spectra Manager software (v 1.53.01).

gels results in the sole presence of the 85 kDa form,<sup>14</sup> supporting the notion that the 80 kDa and 85 kDa cortactin bands represent different conformational isomers. Phosphorylation of cortactin downstream of epidermal growth factor receptor (EGFR) activation results in a shift from 80 kDa to 85 kDa. Under these conditions the 85 kDa cortactin form displays increased serine and threonine phosphorylation,<sup>15</sup> with the “shift” in cortactin  $M_r$  occurring concurrently with ERK1/2-mediated S405 and S418 phosphorylation.<sup>3</sup> Collectively these reports have resulted in proposing that non-phosphorylated cortactin exists in the 80 kDa “closed” form with the carboxyl-terminal SH3 domain binding back upon the proline-rich cortactin domain, blocking the ability of the SH3 domain

binding interface to interact with other ligands. ERK1/2 phosphorylation in turn results in displacing the SH3-proline-rich homotypic cortactin interaction, rendering cortactin in an “open” 85 kDa state where the SH3 domain can bind N-WASP and other cortactin SH3 binding proteins.<sup>3,11,16</sup> These conclusions are supported by chemical crosslinking studies with non-phosphorylated cortactin that indicate cortactin exists as a monomeric globular protein in solution, with the SH3 domain in close proximity to a helical domain amino terminal to the proline-rich region<sup>17</sup> as well as the prevalence of the 85 kDa form in metastatic colorectal carcinoma cases.<sup>18</sup>

We recently analyzed the cortactin “shift” through a combination of phosphorylation-specific antibodies against

cortactin pS405/pS418 and point mutant constructs for these residues. Phosphorylation-specific antibodies confirm the presence of pS405 and pS418 predominantly in the 85 kDa cortactin form in tumor cells following EGF treatment. However, analysis of the cortactin shift with phosphorylation-null point mutants downstream of v-Src mediated ERK1/2 activation reveals a more intricate result. Cortactin S418A and S405A/S418A proteins exhibit the same electrophoretic mobility as wild-type cortactin, with the prominent band at 80 kDa, while a S405A cortactin mutant runs at the shifted 85 kDa  $M_r$ . This could suggest that S418 phosphorylation alone is responsible for driving the 80/85 kDa cortactin shift, with S405 phosphorylation occurring

subsequent to S418 phosphorylation in the 85 kDa form. However, cortactin phosphorylated by ERK1 at pS418 in vitro does not shift from 80 kDa to 85 kDa (Fig. 1A) and ERK1-phosphorylated cortactin does not demonstrate significant alterations in secondary structure compared to non-phosphorylated cortactin when evaluated by circular dichroism (Fig. 1B). These results demonstrate that ERK-mediated cortactin phosphorylation on S405 and S418 is associated with, but is not exclusively responsible for production of the 85 kDa open cortactin form.

We conclude that other factors besides S405/418 phosphorylation are involved in generating and/or maintaining the cortactin shift in EGF-stimulated cells. While the identity of these additional regulatory elements is presently unclear, the proline-rich region where serine 405 and serine 418 reside allows ample opportunity for substantial global conformational alterations through cis-trans isomerization of proline peptide bonds by prolyl isomerases.<sup>19</sup> The compact globular conformation (presumably representative of the closed 80 kDa isomer), with the SH3 domain folding back onto amino-terminal peptide sequences assumed by cortactin in solution<sup>17</sup> is in contrast to the 220 Å-long rod-like cortactin protein characterized by rotary shadowing and electron microscopy<sup>20</sup> that may represent the 85 kDa form. These studies provide indirect

evidence for involvement of the proline-rich region in extensively altering cortactin structure.

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# Oncogenic Src requires a wild-type counterpart to regulate invadopodia maturation

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## Summary

The proto-oncogene Src tyrosine kinase (Src) is overexpressed in human cancers and is currently a target of anti-invasive therapies. Activation of Src is an essential catalyst of invadopodia production. Invadopodia are cellular structures that mediate extracellular matrix (ECM) proteolysis, allowing invasive cell types to breach confining tissue barriers. Invadopodia assembly and maturation is a multistep process, first requiring the targeting of actin-associated proteins to form pre-invadopodia, which subsequently mature by recruitment and activation of matrix metalloproteases (MMPs) that facilitate ECM degradation. We demonstrate that active, oncogenic Src alleles require the presence of a wild-type counterpart to induce ECM degradation at invadopodia sites. In addition, we identify the phosphorylation of the invadopodia regulatory protein cortactin as an important mediator of invadopodia maturation downstream of wild-type Src. Distinct phosphotyrosine-based protein-binding profiles in cells forming pre-invadopodia and mature invadopodia were identified by SH2-domain array analysis. These results indicate that although elevated Src kinase activity is required to target actin-associated proteins to pre-invadopodia, regulated Src activity is required for invadopodia maturation and matrix degradation activity. Our findings describe a previously unappreciated role for proto-oncogenic Src in enabling the invasive activity of constitutively active Src alleles.

**Key words:** Head and Neck cancer, Src, Invadopodia, Cortactin

## Introduction

Src is the first described proto-oncogene and a current target for anti-invasive compounds in clinical trials (Brunton and Frame, 2008; Yeatman, 2004). Src participates in a vast array of cellular functions that include the regulation of cell proliferation, adhesion, migration and invasion (Guarino, 2010; Thomas and Brugge, 1997). In normal cells, Src activity is tightly controlled through intramolecular regulation, subcellular localization and protein expression levels. Elevated or aberrant Src activity is a potent mediator of cell transformation and tumor progression, and is associated with the majority of human cancers including head and neck squamous cell carcinoma (HNSCC) (Summy and Gallick, 2003).

One of the most evident phenotypes of Src-transformed cells is the formation of actin-rich ventral membrane protrusive structures that actively degrade ECM (Chen, 1989). These structures, termed invadopodia, are made by metastatic cancer cells and Src-transformed fibroblasts (Linder, 2009). Invadopodia spontaneously form in tumor cells directly cultured from patient samples and are hypothesized to facilitate breaching of basement membranes during metastasis (Clark et al., 2007; Yamaguchi and Condeelis, 2007). Src activity is absolutely necessary for invadopodia formation and function, and the level of tyrosine phosphorylation at invadopodia positively correlates with the degree of ECM degradation (Bowden et al., 2006; Spinardi et al., 2004). The molecular components that make up invadopodia include proteins that facilitate actin assembly, membrane trafficking and focal degradation. Src substrates participate in all of these functions and include the proteins cortactin

(Bowden et al., 1999), N-WASP (Yamaguchi et al., 2005), dynamin-2 (Baldassarre et al., 2003), AMAP1 (Onodera et al., 2005), paxillin (Bowden et al., 1999), p130Cas (Brabek et al., 2004), Tsk5 (Seals et al., 2005), p190RhoGAP (Nakahara et al., 1998), AFAP110 (Gatesman et al., 2004) and caveolin (Yamaguchi et al., 2009). Several studies have evaluated Src activity in invadopodia formation through the ectopic expression of constitutively active Src alleles (Artym et al., 2006; Oser et al., 2009; Styli et al., 2009). However, these activating Src mutants are rarely found in human tumors, which instead typically contain increased levels of wild-type (WT) Src expression and/or aberrant WT Src activity due to hyperactivation of upstream pathways (Yeatman, 2004). The role of WT Src in invadopodia formation and function is unknown.

Invadopodia assembly has been proposed to involve several stages that regulate the progression from pre-invadopodia (non-degradative) complexes to functional, mature invadopodia containing active MMPs that degrade ECM (Artym et al., 2006; Oser et al., 2009). In current models of invadopodia formation, filamentous (F)-actin and the actin-associated protein cortactin are recruited to sites of matrix adhesion, resulting in pre-invadopodia complexes (Artym et al., 2006). The membrane-bound matrix metalloproteinase MT1-MMP (MMP14) is subsequently recruited to these sites, allowing matrix degradation and invadopodia maturation. Cortactin is an actin-binding protein that is phosphorylated by Src kinase (Head et al., 2003), and is a core invadopodia component. Knockdown of cortactin expression results in decreased invadopodia formation (Artym et al., 2006; Webb et

al., 2007) and MMP secretion (Clark and Weaver, 2008; Clark et al., 2007), whereas phosphorylation of cortactin is important for regulation of matrix degradation at invadopodia (Ayala et al., 2008).

Recent work on discerning the molecular mechanism regulating actin polymerization before MMP recruitment has identified dynamic regulation of cortactin phosphorylation or dephosphorylation downstream of Src to be central to this process (Oser et al., 2009). In this model, cortactin sequesters the actin-severing protein cofilin within pre-invadopodia. Tyrosine phosphorylation of cortactin releases cofilin, which in turn accelerates actin polymerization by severing existing invadopodial actin filaments. In addition, tyrosine phosphorylation of cortactin creates docking sites for the adaptor protein Nck1, which binds and activates the Arp2/3 activator N-WASP. The combined effect of cofilin activation and N-WASP-mediated Arp2/3 activity serves to increase actin polymerization as pre-invadopodia mature and obtain the ability to degrade ECM. Dephosphorylation of cortactin is proposed to stabilize maturing invadopodia by downregulating actin polymerization through liberation of the Nck1–N-WASP complex, coupled with rebinding of inactive cofilin. Although these data implicate cycles of cortactin phosphorylation and dephosphorylation as crucial in invadopodia maturation, it is not known how the phosphorylation of cortactin and other invadopodia maturation-associated proteins is spatially and temporally orchestrated through upstream kinase-based signaling to drive invadopodia maturation. In this study, we have determined that the presence of endogenous, regulated WT c-Src is required for the maturation of pre-invadopodia complexes induced by oncogenic Src activity into degradative invadopodia. In addition, we show that cortactin phosphorylation downstream of WT c-Src is an important mediator of the maturation process.

## Results

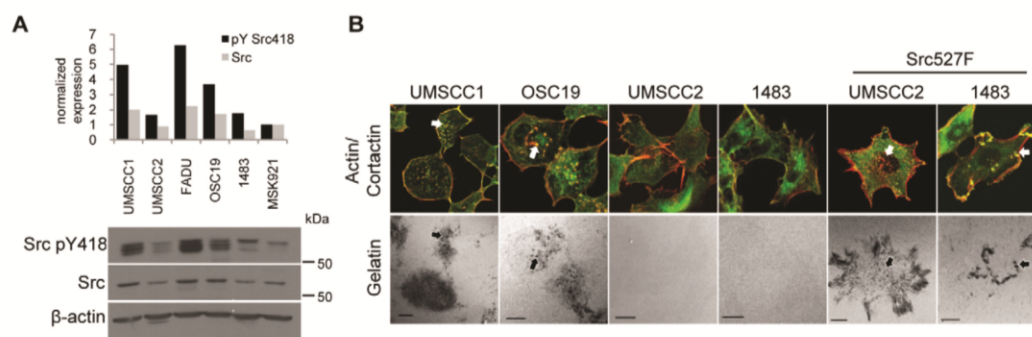
### Elevated Src activity regulates invadopodia formation in HNSCC cell lines

The introduction of constitutively active viral Src (v-Src) or constitutively active cellular Src (Src527F) has been examined in invadopodia formation in cancer cell lines (Artym et al., 2006; Buschman et al., 2009; Oikawa et al., 2008; Styli et al., 2009), but

the role of endogenous c-Src in invadopodia function is unclear. We analyzed a panel of HNSCC lines for endogenous c-Src activity and total c-Src protein levels (Fig. 1A), as well as the ability of these lines to form spontaneous invadopodia on FITC-gelatin matrix (Fig. 1B; supplementary material Fig. S1A). Two (UMSCC1 and OSC19) out of the six tested lines generate invadopodia that were identified by the colocalization of actin and cortactin-rich yellow aggregates in merged images (Fig. 1B, white arrows) coinciding with areas of focal gelatin degradation (black arrows). UMSCC1 and OSC19 cells had substantially elevated c-Src expression and c-Src activity compared with the UMSCC2, 1483 and MSK921 cell lines that do not make invadopodia (Fig. 1A). The FADU cells had elevated c-Src activity, but failed to generate spontaneous invadopodia, suggesting that elevated Src expression alone is not sufficient to drive invadopodia biogenesis in this line. However, invadopodia are formed and matrix degradation occurs in all the HNSCC cell lines that do not form spontaneous invadopodia (UMSCC2, 1483, FADU and MSK921) following exogenous Src527F expression (Fig. 1B; supplementary material Fig. S1B). These data suggest a cell-line-specific threshold of Src activity (highest in FADU cells) that must be reached to support invadopodia formation and matrix degradation. These results are consistent with the idea that elevated Src activity drives invadopodia biogenesis, and are in agreement with previous work with small-molecule Src inhibitors in HNSCC cell lines, which found a dose-dependent decrease of invadopodia formation and matrix degradation (Ammer et al., 2009). Similar results have also been shown for breast cancer cells (Pichot et al., 2009).

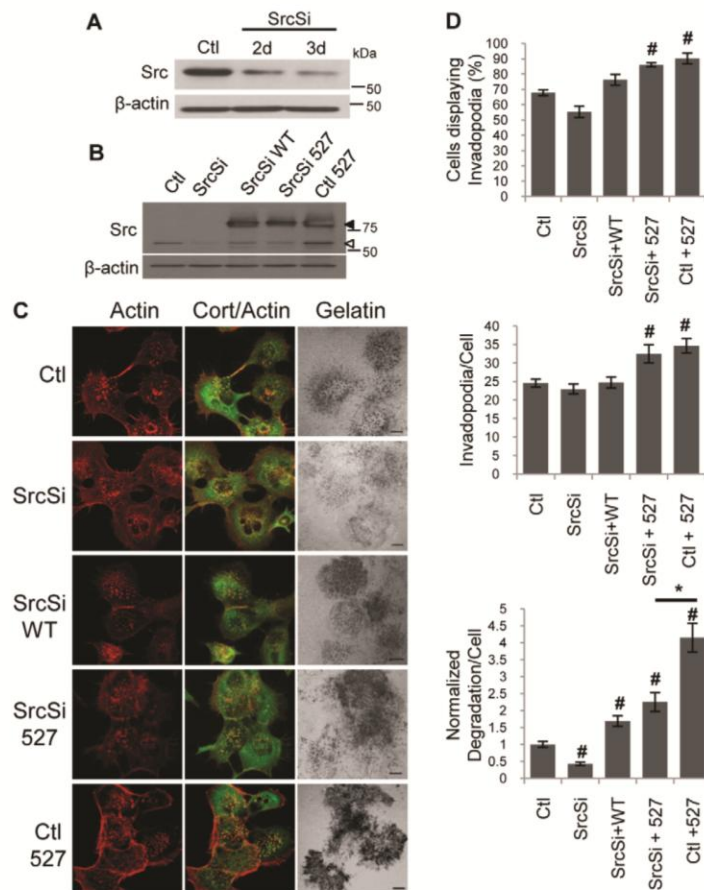
### Endogenous Src expression is required for efficient invadopodia-based matrix degradation in HNSCC cells expressing constitutively active Src

To test the effect of depletion of endogenous c-Src on HNSCC invadopodia formation and function, c-Src expression was knocked down in UMSCC1 cells by RNA interference (SrcSi) (Fig. 2A) and assayed for invadopodia formation and gelatin degradation (Fig. 2B). Endogenous c-Src was depleted by 58% at 2 days, and by 70% at 3 days after transfection. Src-knockdown cells had no statistically significant difference in the number of cells with invadopodia or the number of invadopodia per cell (Fig. 2C, top and middle panels). However, SrcSi cells exhibited a 57% decrease



**Fig. 1. Src activity regulates invadopodia formation in HNSCC lines.** (A) Protein levels of active c-Src (Src-pY<sup>418</sup>), total c-Src (Src) and  $\beta$ -actin (loading control) in HNSCC lines. For quantification of active Src and total Src, expression levels were normalized to MSK921 cells, a line with low Src activity that does not form invadopodia. The multiple bands in the Src-pY<sup>418</sup> panel presumably represent additional Src Family kinases; see Fig. 3A. (B) HNSCC cell lines with or without Src-527F were incubated on FITC-gelatin (pseudocolored white) coverslips for 12 hours and labeled with TRITC-phalloidin (red) and anti-cortactin (green). Invadopodia are identified by the yellow aggregates in the merged images of actin and cortactin (white arrows) that localize with the dark holes in the FITC-gelatin (black arrows). Scale bars: 10  $\mu$ m.





**Fig. 2. Inhibition of endogenous Src expression decreases matrix degradation independently of changes in invadopodia number or increased Src activity.**

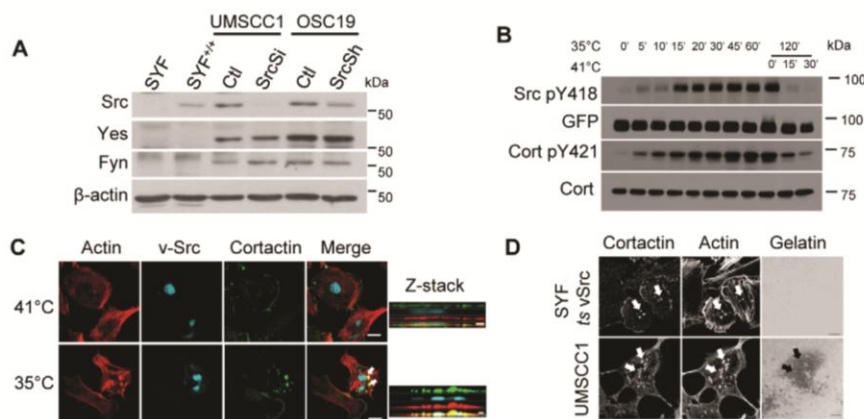
(A) UMSCC1 cells transfected with siRNA directed against Src (SrcSi) or a non-targeting siRNA (Ctl) were evaluated for Src knockdown 2 and 3 days after transfection by anti-Src western blotting.  $\beta$ -actin blotting was used as a loading control. (B) Cell lysates from UMSCC1 cells transfected with non-targeting siRNA (Ctl) or *Src* siRNA (SrcSi) alone, or in combination with cerulean-tagged SrcWT (WT) or Src-527F (527) were evaluated by immunoblotting. Lysates were probed with anti-Src and anti- $\beta$ -actin antibodies. Filled arrowhead indicates exogenously expressed Src (WT or 527F), open arrowhead denotes endogenous Src. (C) Representative confocal images of UMSCC1 cells transfected with non-targeting siRNA (Ctl) or SrcSi alone, or in combination with cerulean-tagged SrcWT (WT) or Src-527F (527). Cells were plated on FITC-gelatin-coated (pseudocolored white) coverslips for 10 hours and immunolabeled with TRITC-phalloidin (red) and cortactin (green). Scale bars: 10  $\mu$ m. (D) Percentage of cells displaying invadopodia (top), the number of invadopodia per cell (middle) and the amount of matrix degradation per cell (bottom) were examined for each line evaluated in B. Data are presented as mean  $\pm$  s.e.m.; #, statistically different from control ( $P \leq 0.01$ ); groups under the bar are statistically different ( $P < 0.01$ ).

in gelatin degradation compared with control cells (Ctl) (Fig. 2C, bottom panel). Rescue of WT Src expression in SrcSi cells (SrcSi+WT) restored matrix degradation to levels above those of the control cells (1.7-fold) (Fig. 2C). Increases above control levels are presumably due to the additive effect of remaining endogenous Src from incomplete knockdown coupled with the modest overexpression of the WT Src construct (Fig. 2B).

We also examined the effect of silencing endogenous c-Src in the presence of constitutively active Src (Fig. 2B,C). Consistently with previous reports (Artym et al., 2006; Oser et al., 2009), expression of Src527F in control cells (Ctl+527) or SrcSi cells (SrcSi+527F) resulted in increases in the number of cells with invadopodia (~30%) and the number invadopodia per cell (~36%). Surprisingly, c-Src knockdown markedly blunted the increased degradation due to Src527F expression, a 2.5-fold (SrcSi+Src527F cells) increase compared with a 4.8-fold (Ctl+527F) increase over control cells (Fig. 2C). These results demonstrate that constitutively active Src cannot completely rescue the role of endogenous c-Src in regulation of matrix degradation. Collectively, these data indicate that although increased Src activity enhances invadopodia formation, the presence of endogenous c-Src is required for optimal matrix degradation. Similar results were found in OSC19 cells (supplementary material Fig. S2).

#### Constitutively active Src is sufficient to promote invadopodia formation but not ECM degradation in *Src*-null cells

Complete c-Src knockdown in our HNSCC lines is technically problematic because additional Src family kinases (Yes and Fyn) with unknown functions in invadopodia biology are present and maintained in OSC19 and UMSCC1 cell lines treated with SrcSi (Fig. 3A). We therefore used Src-, Yes- and Fyn-deficient (SYF) fibroblasts to further evaluate the role of WT Src in invadopodia function. A GFP-tagged temperature-sensitive mutant of v-Src (tsLa29-GFP) was generated and expressed in SYF cells to dynamically regulate Src activity and invadopodia formation. Src kinase activation occurs within 15 minutes when cells are switched from the non-permissive temperature (41°C) to the permissive temperature (35°C) (Fig. 3B). v-Src inactivation occurs within 30 minutes when cells are shifted back to 41°C. Activation of v-Src leads to the phosphorylation of cortactin on Tyr421, indicating that tsLa29-GFP regulates phosphorylation of a known downstream Src target that is crucial for invadopodia assembly. In agreement with previous reports (Walker et al., 2007), invadopodia formation is induced when cells expressing tsLa29-GFP are switched to the permissive temperature (Fig. 3C). These invadopodia are enriched with active v-Src and phosphorylated cortactin (Fig. 3C;



**Fig. 3. Invadopodia produced by constitutively active Src in SYF fibroblasts fail to degrade ECM.** (A) Src family kinase expression in HNSCC and fibroblast cell lines. Clarified cell lysates from SYF, SYF<sup>+/+</sup>, UMSCC1 (treated with control and SrcSi), and OSC19 (expressing control vector and *Src* shRNA) cells were resolved by SDS-PAGE and immunoblotted with anti-Src, anti-Yes, anti-Fyn and anti-β-actin antibodies. (B) Time course of tsLa29 v-Src activation and resulting cortactin phosphorylation. Cells transfected with tsLa29 were incubated at 35°C (permissive temperature) for the indicated times and were lysed or returned to 41°C (non-permissive temperature) for 15 or 30 minutes and analyzed for Src-pY<sup>418</sup>, GFP, cortactin, and cortactin-pY<sup>421</sup>. (C) Invadopodia formation in cells expressing v-Src. SYF cells were transfected with tsLa29-GFP (pseudocolored light blue) and incubated at 41°C or 35°C and labeled with TRITC-phalloidin (red) and cortactin (green). Cells were visualized by confocal microscopy and z-stack sectioning. Invadopodia are visible in z-stack images as actin- and cortactin-rich puncta that are several micrometers in length (white arrows). Scale bars: 10 μm, 5 μm (z-stacks). (D) Defective ECM degradation in SYF cells expressing v-Src. SYF cells transfected with tsLa29-v-Src and non-transfected UMSCC1 cells (positive control) were plated directly onto FITC-gelatin-coated coverslips. After 24 hours, cells were labeled with TRITC-phalloidin and anti-cortactin antibodies. White arrows indicate invadopodia and black arrows, areas of degraded matrix in C and D. Scale bars: 10 μm.

supplementary material Fig. S3A). As reported in other invadopodia-forming cell systems (Artym et al., 2006; Ayala et al., 2008; Clark et al., 2007; Webb et al., 2006), inhibition of cortactin expression using siRNA diminishes the ability of tsLa29-GFP to induce invadopodia formation at the permissive temperature (supplementary material Fig. S3B,C).

To evaluate the functionality of invadopodia in this system, we plated SYF cells on FITC-gelatin-coated coverslips to assay ECM degradation. Invadopodia induced by tsLa29-GFP in the *Src*-null fibroblasts fail to degrade the ECM at periods up to and beyond 48 hours, indicating they remain in a pre-invadopodia state (Fig. 3D). Experiments with an untagged v-Src produced a similar result, ruling out improper activation or localization related to the addition of GFP. We also attempted to rescue invadopodia maturation in the SYF cells with tsLa29 v-Src. Manipulation of tsLa29 v-Src activity over the 24 hour incubation period by switching cells from permissive to the non-permissive temperatures did not result in invadopodia maturation (data not shown). These results demonstrate that constitutively active v-Src activity is responsible for the induction of the initial phosphorylation cascade that drives recruitment of invadopodia components to form pre-invadopodia complexes, but these v-Src-induced complexes are insufficient to direct matrix degradation in SYF cells.

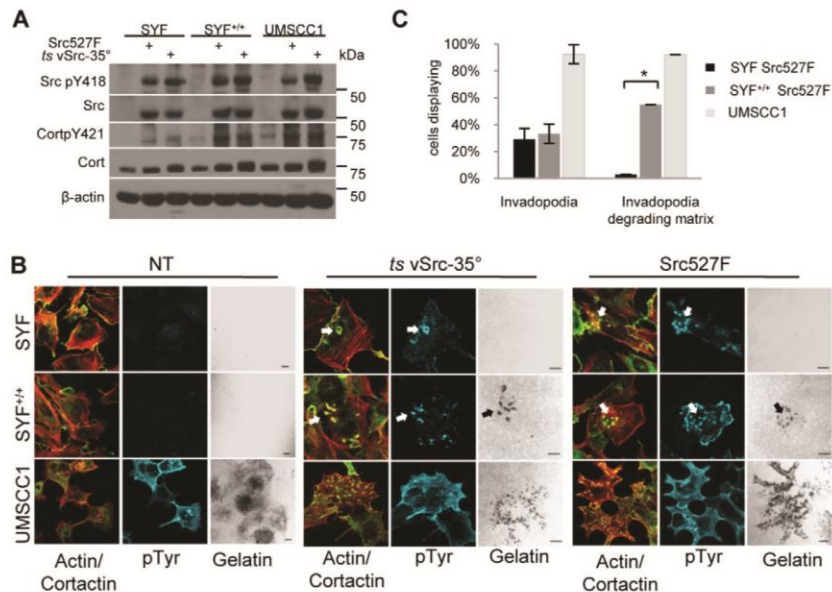
#### Wild-type Src kinase is necessary for invadopodia maturation

Based on our findings we hypothesized that either: (1) another ubiquitously expressed Src family kinase (Yes and/or Fyn) absent from SYF cells is required for invadopodia maturation in addition to active Src, or (2) WT Src or 'regulated' Src must also be present with active Src for degradation of ECM to occur. To test these hypotheses, we used a SYF cell line with two copies of WT Src

genetically reintroduced to restore normal WT Src expression (SYF<sup>+/+</sup>; Fig. 3A, Fig. 4A). The percentage of cells forming invadopodia and the percentage of invadopodia-forming cells degrading matrix were assessed following introduction of v-Src or Src527F. Transfection with activated Src constructs in the form of tsLa29 or Src527F induces mature matrix-degrading invadopodia in SYF<sup>+/+</sup> cells, in contrast to pre-invadopodia formation in SYF cells (Fig. 4B). Approximately 55% of SYF<sup>+/+</sup> cells forming invadopodia contained invadopodia that actively degraded matrix, compared with 2% of SYF cells (Fig. 4C). There was no difference in the percentage of cells forming invadopodia (pre and mature) in SYF or SYF<sup>+/+</sup> cells expressing Src527F (Fig. 4C), which is similar to results in UMSCC1 cells (Fig. 2C). Also, the level of general phosphotyrosine-containing proteins localized to invadopodia was unchanged in SYF527F and SYF527F<sup>+/+</sup> cells (Fig. 4B). Taken together, these results suggest that catalytically active Src alone promotes the assembly of pre-invadopodia complexes and targets tyrosine phosphorylation of proteins within these structures, but SrcWT is necessary for pre-invadopodia maturation required to induce ECM degradation.

To confirm these findings, WT Src expression was transiently restored in SYF cells and assayed for ECM degradation. Monitoring of the coexpression of Src527F and SrcWT was achieved by creating C-terminal linker fusions with mCherry (Src527F-mCherry) and cerulean (Src-Cer) fluorescent proteins (supplementary material Fig. S4). Transfection efficiency of the co-transfected Src constructs was consistently greater than 90% and imaging revealed that nearly all SYF cells expressed both Src alleles (supplementary material Fig. S5A). In SYF cells expressing SrcWT alone, Src had a perinuclear localization that was consistent with previous reports (Sandilands et al., 2004) (supplementary material Fig. S4B,C). Coexpression of Src527F with WT Src





**Fig. 4. WT Src rescues invadopodia maturation in cells expressing constitutively active Src.** (A) SYF, SYF<sup>+/+</sup> and UMSSC1 cells that were non-transfected (NT), transfected with Src527F or ts-v-Src, were lysed, resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src clone EC10 (only recognizes avian Src), anti-cortactin-pY421, anti-cortactin (4F11), and anti-β-actin antibodies. (B) SYF, SYF<sup>+/+</sup> and UMSSC1 cells that were non-transfected (NT), transfected with SRC-527F, or tsLa29-v-Src were plated onto FITC-conjugated gelatin coverslips and evaluated by confocal microscopy. Cells were labeled with TRITC-phalloidin (red), cortactin (green), and anti-phosphotyrosine (light blue) antibodies. Invadopodia are identified by the yellow aggregates in the merged image of actin and cortactin in cells containing phosphotyrosine. In addition, degrading (mature) invadopodia localize with the dark holes (arrows) in the FITC-gelatin (white). (C) Quantification of the percentage of cells forming invadopodia structures (actin and cortactin aggregates, left), and the percentage of invadopodia-forming cells that contain matrix degradation (mature invadopodia, right). Data are represented as mean ± s.d.; \* $P \leq 0.05$ .

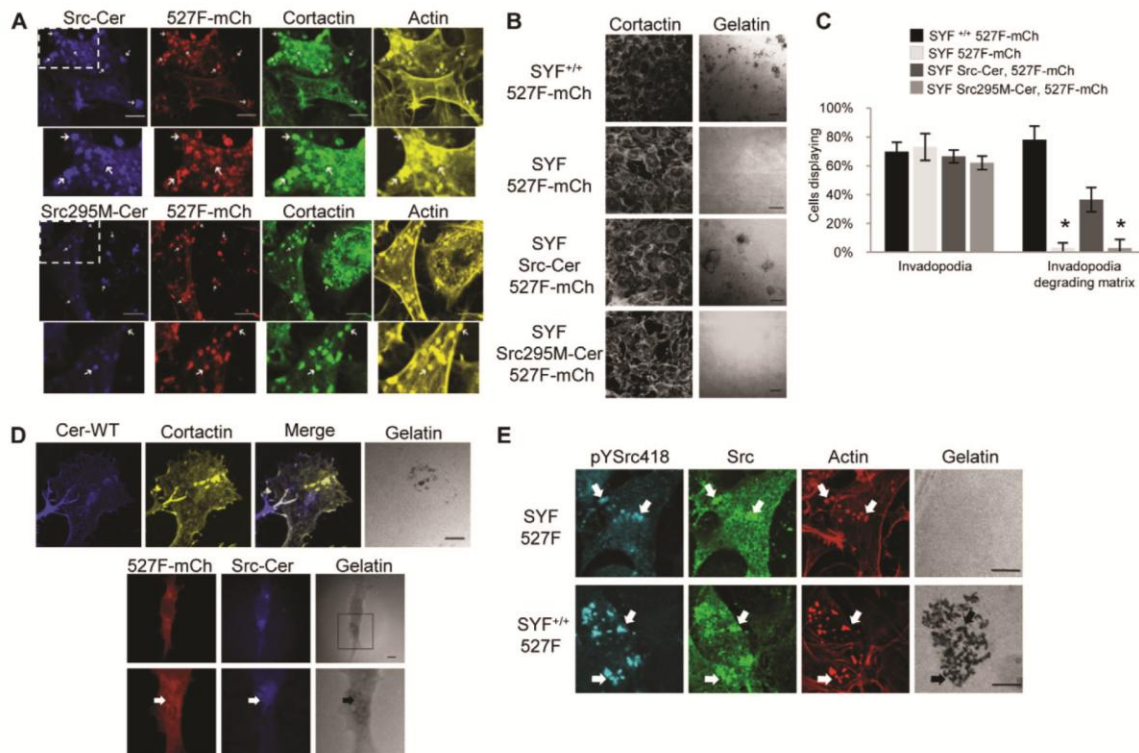
results in recruitment of WT Src to invadopodia where it colocalizes with Src527F (Fig. 5A). In addition, cells coexpressing these constructs regain the ability to degrade ECM (Fig. 5B,C). To further verify the functional requirement for WT Src in invadopodia maturation, we conducted WT-Src-specific staining of SYF cells containing Src527F. WT Src colocalizes with cortactin to areas of ECM degradation, further demonstrating that Src localizes to mature invadopodia (Fig. 5D, top panels). In addition, direct visualization of WT Src-Cer and Src527F-mCherry in SYF cells demonstrates a concentration of Src-Cer at areas of ECM degradation, with Src527F-mCh localized to the same vicinity (Fig. 5D, bottom panels).

Since the localization of endogenous Src to invadopodia is required for invadopodia maturation, we determined whether catalytically inactive Src could substitute for WT Src and rescue ECM degradation in SYF cells. This result would suggest that two separate and distinct pools of Src (constitutively active and kinase-inactive) are necessary and sufficient for maturation. To test this hypothesis, constitutively active Src527F-mCherry was coexpressed with a cerulean-tagged kinase-inactive Src (Src295M-Cer) in SYF cells. Similarly to WT Src, Src295M was largely perinuclear when expressed alone in SYF cells (supplementary material Fig. S4B,C), but was recruited to invadopodia when coexpressed with Src527F (Fig. 5A). However, Src295M failed to rescue invadopodia maturation (Fig. 5B,C), indicating that catalytically inactive Src does not substitute for WT Src function. Since constitutively active and kinase-dead Src cannot support

invadopodia maturation, this suggests that WT Src kinase activity is dynamically regulated to promote invadopodia maturation. Similarly, cell staining for active Src (pY418) localized to pre-invadopodia in SYF cells and mature invadopodia in SYF<sup>+/+</sup> cells (Fig. 5E), ruling out the possibility that catalytically active Src initially drives invadopodia assembly, then is inactivated and remains inactive during maturation. Taken together, these results demonstrate that regulated WT Src kinase activation and inactivation within pre-invadopodia complexes is necessary to govern the downstream signaling events required for invadopodia maturation and ECM degradation.

#### Regulated Src activity directs cortactin phosphorylation dynamics to control invadopodia maturation

In proposed models of invadopodia maturation, dynamic cortactin tyrosine phosphorylation is required for pre-invadopodia maturation (Oser et al., 2009). To identify a potential mechanism of invadopodia maturation affected by regulated Src activation and inactivation, we assessed the role of Src kinase activity on cortactin phosphorylation. In the SYF/SYF<sup>+/+</sup> system, cortactin phosphorylated on Tyr421 localized to pre-invadopodia and mature invadopodia (Fig. 6A), suggesting that cortactin phosphorylation is important in pre-invadopodia assembly and invadopodia maturation. This is consistent with a well-described role for cortactin in invadopodia maturation in other systems (Artym et al., 2006; Clark et al., 2007; Oser et al., 2009). Stable cell lines were generated expressing endogenous levels of WT cortactin (control)



**Fig. 5. Expression of regulated Src is necessary for ECM degradation at invadopodia.** (A) Confocal imaging of SYF cells coexpressing Src-Cerulean and Src527F-mCherry, or Src295M-Cerulean and Src527F-mCherry. Cells were immunolabeled with anti-cortactin and Alexa-Fluor-647-labeled phalloidin. Dashed boxes represent enlarged image regions shown below. Arrows denote invadopodia. (B) Representative fields of SYF and SYF<sup>+/+</sup> cells expressing Src527F-mCherry, SYF cells coexpressing Src527F-mCherry and Src-Cerulean, or SYF cells coexpressing Src527F-mCherry and Src295M-Cerulean. Cells were plated on FITC-gelatin-coated coverslips for 24 hours and immunolabeled with anti-cortactin. Areas of gelatin degradation and clearing appear black against the pseudocolored white background. (C) Percentage of cells with forming invadopodia (actin and cortactin aggregates, left), and percentage of invadopodia-forming cells with degraded matrix (mature invadopodia, right) from the experimental conditions shown in B. Data are represented as mean  $\pm$  s.d.; \* $P \leq 0.05$ . (D) Confocal imaging (top) or swept-field imaging (bottom) of SYF cells coexpressing c-Src527F and Src-Cerulean. Cells were plated on FITC-gelatin-coated coverslips and immunolabeled with anti-cerulean (dark blue) and anti-cortactin (yellow) (colocalization appears white in merged image; top panel) or directly imaged for Src527F-mCherry (red) and Src-Cerulean (dark blue) (bottom panels). Arrow indicates colocalization of Src 527F-mCherry and WT Src-Cerulean at sites of gelatin degradation. (E) Confocal imaging of Src activity in SYF and SYF<sup>+/+</sup> cells expressing Src527F. Cells were incubated on FITC-gelatin-coated coverslips for 24 hours, fixed and immunolabeled with TRITC-phalloidin (red), anti-pY418 Src (light blue) and anti-Src (green) antibodies. White arrows denote invadopodia; black arrows, matrix degradation. Scale bars: 20  $\mu$ m (B), 10  $\mu$ m (A,D,E).

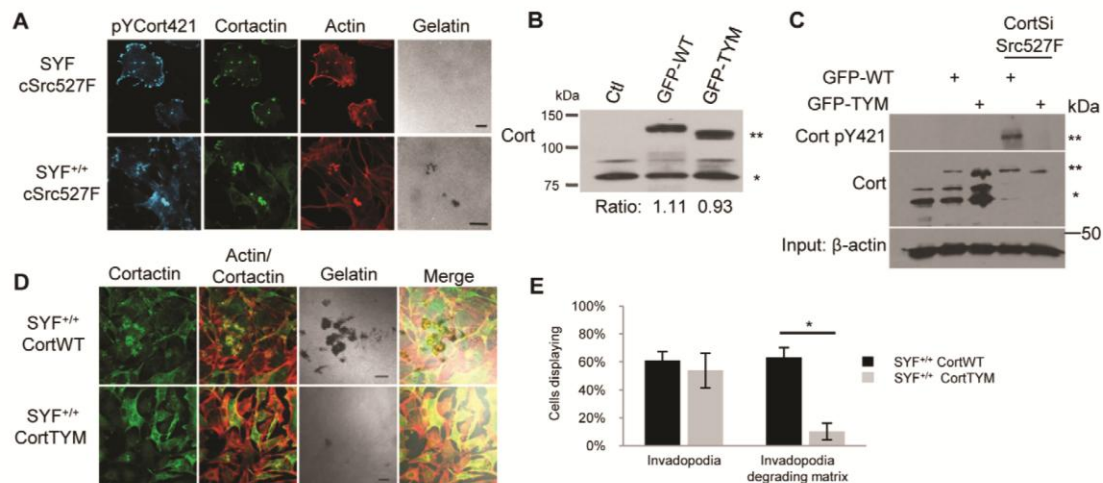
or a cortactin mutant in which the three Src-targeted tyrosine residues (421, 470, 486) are mutated to phenylalanine (SYF<sup>+/+</sup> CortTYM) (Fig. 6B). Endogenous murine cortactin was silenced with siRNA (CortSi, knockdown >90%), resulting in the exclusive expression of WT or mutant human cortactin (Fig. 6C). Wild-type control and CortTYM SYF<sup>+/+</sup> cell lines treated with siRNA to knock down cortactin were transfected with Src527F to promote invadopodia formation. Expression of WT human cortactin rescued the inhibitory effects of cortactin knockdown on invadopodia formation, resulting in the formation of mature invadopodia (~60% of total cells with invadopodia, Fig. 6D,E). However, only ~10% of SYF<sup>+/+</sup> CortTYM cells produced degrading invadopodia. Consistent with previous reports (Oser et al., 2009), there were no differences observed in the percentage of cells forming actin or cortactin aggregates in control and CortTYM SYF<sup>+/+</sup> cells, demonstrating that cortactin is targeted to pre-invadopodia

independently of tyrosine phosphorylation. However, expression of mutant cortactin in SYF<sup>+/+</sup> cells completely blocked the upstream function of WT Src, rendering the SYF<sup>+/+</sup> Src527F cells with a degradation profile that was similar to that seen in cells lacking WT Src (SYF Src527F, Fig. 4B,C). These results indicate that there is differential phosphotyrosine signaling in SYF cells with WT Src that supports invadopodia maturation downstream of constitutively active Src.

#### Cells forming pre- and mature invadopodia have distinct phosphotyrosine signatures

To examine whether distinct tyrosine phosphorylation signatures occur in cells that form pre-invadopodia and mature invadopodia, we conducted a non-biased, comprehensive and quantitative SH2-domain screen (Machida et al., 2007) to identify differences in potential phosphotyrosine-binding proteins under conditions of





**Fig. 6. WT Src regulates cortactin phosphorylation during invadopodia maturation.** (A) SYF and SYF<sup>+/+</sup> cells expressing Src527F were incubated on FITC-gelatin-coated coverslips for 24 hours, fixed and immunolabeled with TRITC-phalloidin (red), human anti-pY421 cortactin (light blue) and anti-cortactin (green) antibodies. Note that cortactin is phosphorylated in pre-invadopodia and in mature invadopodia, as defined by the absence or presence of matrix degradation. (B) Validation of GFP-tagged cortactin expression in stable cell lines. Cellular extracts from SYF or SYF<sup>+/+</sup> cells stably expressing GFP-CortWT or GFP-CortTYM were resolved by SDS-PAGE and immunoblotted with an anti-cortactin (4F11) antibody. \*, endogenous cortactin; \*\*, GFP-tagged cortactin (the slight mobility disparity is due to the size difference in AcGFP and EGFP tags); ratios of exogenous (WT and TYM) to endogenous cortactin is depicted under the blot. (C) Clarified lysates from SYF<sup>+/+</sup> GFP-CortWT and SYF<sup>+/+</sup> GFP-CortTYM cells were transfected with *Cttm* siRNA alone or in combination with Src527F. Cortactin was immunoprecipitated with the anti-cortactin (4F11) antibody, immunocomplexes resolved by SDS-PAGE and immunoblotted with human anti-pY421 cortactin and anti-cortactin (4F11) antibodies. Total cell lysates were immunoblotted with anti-β-actin as a loading control. (D) SYF<sup>+/+</sup> cells stably expressing human GFP-CortWT or GFP-CortTYM were transfected with murine cortactin-targeted siRNA to eliminate endogenous cortactin expression. Two days later cells were transfected with Src527F and plated on FITC-gelatin-coated coverslips for 24 hours to promote gelatin degradation. (E) Cells were assessed for the percentage of cells forming invadopodia (actin and cortactin aggregates) and the percentage of invadopodia-forming cells with matrix degradation. Data are represented as mean ± s.d., \**P* < 0.05. Scale bars: 10 μm (A), 20 μm (B).

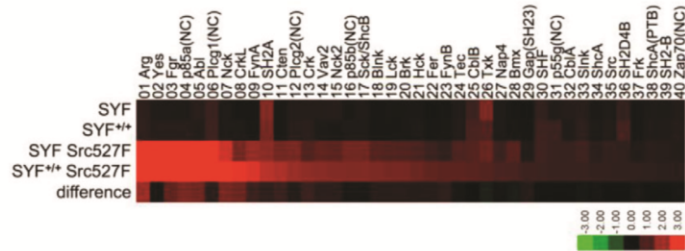
pre-invadopodia and mature invadopodia formation (Fig. 7; supplementary material Fig. S6). Non-transfected SYF and SYF<sup>+/+</sup> cells had minimal differences in SH2-domain binding profiles. Expression of Src527F in either cell type enhanced overall SH2 domain signal-binding intensity, indicating a broad increase in phosphotyrosine signaling and creation of new SH2-domain docking sites. This result was anticipated with expression of constitutively active Src. However, cells that form pre-invadopodia (SYF Src527F) had distinct differences in their SH2 binding intensity from cells that form functional mature invadopodia (SYF<sup>+/+</sup> Src527F) (bottom row, difference). The variations in binding intensity that arise in SYF and SYF<sup>+/+</sup> cells expressing Src527F indicate a fundamental mechanistic difference in the concentration of phosphotyrosine binding sites for several SH2-domain-containing proteins that are likely to have key roles in invadopodia maturation. Interestingly, tyrosine-phosphorylated cortactin is known to interact with several high-intensity 'hits', including Arg, Abl (Boyle et al., 2007), Fer (El Sayegh et al., 2005), Crk (Bougneres et al., 2004) and Nck (Tehrani et al., 2007), consistent with its role in invadopodia maturation. Experiments to elucidate additional proteins involved in these signaling complexes are currently underway.

## Discussion

In this study, we investigated the role of endogenous or WT c-Src, and the interplay between constitutively active Src and cellular Src in invadopodia formation. Previous studies on Src in invadopodia

formation have exclusively manipulated Src activity in cells containing WT c-Src. Tumor cells that form spontaneous invadopodia presumably have upstream oncogenic signals such as overactivation or overexpression of epidermal growth factor receptor (EGFR), which drives c-Src activation (Xue et al., 2006). Accordingly, increased Src kinase activity (through overexpression of constitutively active Src, or overexpression of WT Src) in tumor cells that form spontaneous invadopodia is associated with increased invadopodia formation and matrix degradation (Artym et al., 2006; Oser et al., 2009). Unlike previous reports (Oser et al., 2009), we did not find that tumor cells overexpressing constitutively active Src had less degradation per invadopodia than spontaneous invadopodia formed in control cells (Fig. 2; supplementary material Fig. S2). However, these differences might be attributed to a much larger capacity for invadopodia formation in UMSSC1/OSC19 cells compared with MTLn3 cells (~25 vs ~two invadopodia formed in control cells, respectively). In HNSCC cells and Src-null fibroblasts, we propose that constitutively active Src acts as an oncogenic 'trigger' that promotes pre-invadopodia formation, whereas WT Src acts downstream to direct invadopodia stability and maturation. Although models of spontaneous invadopodia formation are invaluable to the field, our finding that Src cycling is essential for invadopodia maturation could only be completely uncovered through the use of the SYF/SYF<sup>+/+</sup> system.

In this study, we show that WT Src is indispensable for invadopodia maturation driven by elevated Src activity. We hypothesize that distinct, temporally and spatially regulated Src



**Fig. 7. Enhanced binding of SH2 domains binding in SYF<sup>+/+</sup> cells expressing Src527F.** In vitro SH2 and PTB domain-binding profiles for SYF, SYF<sup>+/+</sup> cells, and SYF and SYF<sup>+/+</sup> cells expressing Src527F. Binding of SH2 or PTB domains to cell lysates are shown as a heat map where intensity of red indicates strength of binding. Domain names are on the top row, with a rank order of binding to SYF<sup>+/+</sup> Src527F to denote potential importance in invadopodia maturation. Sample labels are on the left side. Difference=Signal[SYF<sup>+/+</sup> Src527F]-Signal[SYF Src527F].

function is necessary to regulate the phosphorylation of cortactin. It is likely that Src regulates other scaffolding proteins that are important in invadopodia stability and maturation, such as dynamin (Caldieri et al., 2008), Tks5 (Seals et al., 2005), IQGAP1 (Sakurai-Yageta et al., 2008) and paxillin (Bowden et al., 1999). It is possible that Src is activated before its localization with downstream actin-associated substrates in pre-invadopodia. Once pre-invadopodia assembly is complete, Src is inactivated [presumably by C-terminal Src kinase (CSK) acting on pY527 (Okada et al., 1991)] and released to regulate additional proteins involved in actin dynamics and MMP delivery to invadopodia. In this way, Src might act as a regulator of several sequentially coordinated protein-interaction events that direct the diverse array of cellular processes at invadopodia, including actin assembly, membrane trafficking and ECM degradation.

It is known that invadopodia produced by cancer cells and transformed fibroblasts use similar signaling pathways and contain many of the same proteins as podosomes produced by osteoclasts, dendritic cells and macrophages (Linder, 2009). SRC-null osteoclasts have been used to study the role of Src in podosome assembly and dynamics (Destaing et al., 2008). WT Src and Src527F were both able to restore normal podosome organization, whereas Src295M did not. These data in podosomes support our findings in invadopodia, but since this report did not address the functionality of these cells to degrade ECM, it is not known whether WT Src is needed for maturation in this system. These results are particularly interesting because Src has been suggested to act as a 'molecular switch' to regulate dynamin-Cbl signaling complexes (Bruzaniti et al., 2005) and as a protein with an important (kinase independent) adaptor function (Bruzaniti et al., 2009) in osteoclast podosomes. Manipulation of Src activity in WT osteoclasts also suggest a dual function for Src in the regulation of actin dynamics through cortactin phosphorylation in podosome assembly and subsequent maturation into more highly organized structures known as sealing zones (Luxenburg et al., 2006).

We identify cortactin as a key regulator of invadopodia maturation downstream of WT Src activity. We show that constitutively active Src is sufficient to target cortactin to pre-invadopodia (Fig. 3C, Fig. 4B), but cortactin is targeted independent of its tyrosine phosphorylation (Fig. 6D,E). This is in agreement with previous reports demonstrating that dephosphorylated cortactin acts as a scaffold to recruit Arp2/3, N-WASP and cofilin (Oser et al., 2009). Interestingly, cortactin phosphorylated at Tyr421 is enriched in pre-invadopodia (SYF Src527F) and in matrix-degrading mature invadopodia (SYF<sup>+/+</sup> Src527F) (Fig. 6A; supplementary material Fig. S3A). In addition, tyrosine phosphorylation of cortactin is required for invadopodia maturation and ECM degradation (Fig. 6D,E) (Ayala et al., 2008; Desmarais et al., 2009; Webb et al., 2007). These data suggest that

phosphorylation of cortactin occurs before ECM degradation. However, in our SYF/SYF<sup>+/+</sup> model this initial cortactin phosphorylation is not sufficient to drive maturation in the absence of WT Src, suggesting that cortactin must be dephosphorylated before maturation can occur. Our proposed mechanism of dynamic Src activity on cortactin phosphorylation in invadopodia maturation is consistent with the model recently proposed by the Condeelis laboratory (Oser et al., 2009), whereby cyclical cortactin phosphorylation regulates actin polymerization and invadopodia stabilization.

Here, we show that Src activation and inactivation regulates cortactin phosphorylation during invadopodia maturation. Future studies will be required to determine whether Src kinase is directly responsible for the initial phosphorylation of cortactin to promote invadopodia assembly or in subsequent step(s) to regulate maturation (Huang et al., 1998). Fer (El Sayegh et al., 2005) and Abl family kinases (Boyle et al., 2007) are also present in SYF cells and might act downstream of Src to regulate one or more of these steps. It also follows that tyrosine phosphatases are critically important in the regulation of invadopodia maturation, because cortactin and other possible targets downstream of Src kinase activity in our system require dynamic cycles of phosphorylation and dephosphorylation to drive ECM degradation. Protein tyrosine phosphatase 1B is a good candidate for this process because it is known to regulate Src in invadopodia dynamics (Cortesio et al., 2008) and has subsequently been shown to regulate cortactin dephosphorylation (Stuible et al., 2008). Nonetheless, we show that WT Src kinase is the critical upstream regulator of other downstream kinases and phosphatases important to these processes.

Invadopodia maturation is associated with the delivery of MMPs to invadopodia sites (Artym et al., 2006; Clark et al., 2007). MT1-MMP is cited as the key MMP that regulates ECM degradation at invadopodia (Poincloux et al., 2009) and Src kinase activity is known to regulate phosphorylation of MT1-MMP and proteins associated with its trafficking to the cell membrane (Nyalendo et al., 2008; Nyalendo et al., 2007). Future studies should address whether WT Src is necessary to control trafficking to, or activation of MT1-MMP at invadopodia. These studies are further warranted because it has been proposed that a major role of cortactin in invadopodia function involves the targeting and delivery of MMPs to invadopodia to enhance ECM degradation (Clark and Weaver, 2008; Clark et al., 2007).

This report identifies a dominant oncogene that requires the proto-oncogenic complement to reach its full spectrum of transforming functionality. Unlike tumor suppressor proteins, which often require loss of non-mutated alleles, little is known about the potential effect of the presence or absence of proto-oncogenes on their oncogenic counterparts during tumorigenesis. A function in Ras-induced tumorigenesis has been described for Ras proto-



oncogenes (Singh et al., 2005), where WT Ras has been reported to regulate the functioning of oncogenic Ras with regards to cell proliferation. Our results are consistent with those from the majority of solid human tumors that contain elevated c-Src expression or specific activity due to hyperactivation of upstream regulatory pathways, rather than activating point mutations (Yeaman, 2004). To our knowledge, this is the first description of such a finding. Similar mechanisms might be required for other transforming kinases involved in driving tumor progression.

## Materials and Methods

### Cell culture

HNSCC cell lines UMSSC1, FADU and OSC19 were obtained from Jeffery Myers (M. D. Anderson Cancer Center, Houston, TX). These lines along with UMSSC2 (Thomas Carey, University of Michigan, Ann Arbor, MI), 1483 (David Raben, University of Colorado Denver, Aurora, CO) and MSK921 (Peter Sacks, New York University, New York, NY) were cultured as previously described (Rothschild et al., 2006). SYF (Src<sup>-/-</sup>Yes<sup>-/-</sup>Fyn<sup>-/-</sup>) and SYF<sup>+/+</sup> (Src<sup>+/+</sup>Yes<sup>+/+</sup>Fyn<sup>+/+</sup>) cells were obtained from the American Type Culture Collection. Control non-silencing and human SRC-targeted siRNA (5'-AAACTCCCTTGGCTCATGTACTT-3') were from Dharmacon. OSC19 cells stably expressing vector control or SRC-targeted shRNA were created by infection with control or human Src specific lentivirus (Santa Cruz) and cultured according to the manufacturer's instructions. SYF or SYF<sup>+/+</sup> cells stably expressing GFP-tagged human cortactin {WT or Y421F, Y466F, Y482F [triple point mutant (TPM)]} were created using the Flp-In<sup>TM</sup> system (Invitrogen). These lines were transfected with siRNA targeting murine cortactin (*Cttm*) (5'-GCTTCGAG-AGAATGCTTC-3') (siCTTN, Dharmacon). For transient transfections, 3 × 10<sup>6</sup> cells were incubated with 2 µg plasmid construct or siRNA. Fibroblast lines were transfected with the Nucleofector I device (Amaxa Biosystems) and HNSCC lines were transfected with TurboFect<sup>TM</sup> transfection reagent (Fermentas).

### Immunofluorescence labeling and confocal microscopy

Cells were plated on FITC-gelatin (Sigma)-coated coverslips as described (Artym et al., 2006) for 12–24 hours. Cells were fixed with fresh 4% formaldehyde and permeabilized with 0.4% Triton X-100 in PBS. Primary antibodies were diluted with 5% BSA in PBS. Antibodies used were: cortactin 4F11, cortactin EP1922Y (Novus Biologicals), human cort-pY421 (Ammer et al., 2009), Src-pY418 (Biosource), Src GD11 (Upstate), GFP/Cerulean 3E6 (Invitrogen), pTyr-100 (BD Biosciences), Alexa Fluor 405 goat anti-rabbit and Alexa Fluor 647 goat anti-mouse (Molecular Probes). F-actin was labeled with Rhodamine-conjugated phalloidin (Molecular Probes). Cells were mounted in Fluoromount-G (Southern Biotech) and imaged with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss Microimaging). A Nikon Swept-Field using Nikon Elements software (Nikon Instruments) was used for imaging of Src-Cer.

### Invadopodia and matrix-degradation assays

Cells with invadopodia were identified by the presence of at least one actin and cortactin aggregate within the cell ( $n \geq 300$ ). The number of invadopodia per cell was calculated by counting the number of actin and cortactin aggregates within invadopodia forming cells ( $n \geq 150$ ). Degradation per cell area was analyzed using ImageJ as described previously (Clark et al., 2007). Cells with invadopodia-degrading matrix were identified by at least one actin and cortactin aggregate colocalizing with a 'dark hole' that corresponded to degraded FITC-matrix ( $n \geq 50$ ). Data were pooled from several independent experiments;  $n$  represents the number of cells analyzed within each experimental group.

### Antibodies and western blotting

Western blotting of cell lysates was conducted as described (Rothschild et al., 2006). The following antibodies were used: 4F11, Src clone GD11 (Upstate);  $\beta$ -actin (Calbiochem); Living Colors GFP clone JL-8 (BD); Cort-pY421, Src-pY418 (Biosource); avian Src clone EC10 (Millipore) and Yes, Fyn (Cell Signaling).

### Plasmids

The Src-GFP linker constructs (WT, 527F, and 295M) were a gift from Margaret Frame (The Beatson Institute for Cancer Research, Glasgow, UK). Substitution of green fluorescent protein (GFP) with cerulean or mCherry fluorescent protein was accomplished through digestion of Src-pEGFP-N1, pmCherry-C1, and mCerulean-C1 fluorescent vectors with *AgeI* and *BrsGI*. The resulting mCherry and mCerulean fragments (~700 bp) were ligated into the GFP-digested Src-containing pEGFP-N1 vector (BD). Temperature-sensitive v-Src (tsLA29) was subcloned from pCMV-tsLA29 vector into EGFP-N1 using unique *EcoRI* and *BamHI* restriction sites.

### Human cortactin constructs.

A single-stranded primed cDNA library (Invitrogen) was used for cloning human cortactin (*CTTN*) cDNA. The cDNA was PCR amplified to produce a 965 bp *KpnI*–*HincII* fragment and a 688 bp *HincII*–*EcoRI* fragment. Fragments were ligated into

pcDNA3FLAG2AB that had been digested with *KpnI* and *EcoRI*, to generate the 1653 bp full-length *CTTN* cDNA. The *CTTN* triple tyrosine mutant (TYM) was generated using the QuikChange kit with primers designed to alter codons 421, 470 and 486 from tyrosine to phenylalanine and confirmed by DNA sequencing. WT and TYM *CTTN* cDNAs were subsequently amplified as *EcoRI*–*KpnI* fragments and subcloned into pEGFP-N1 (WT) or pAcGFP-N1 (TYM). GFP-CTTN WT and TYM fragments were amplified by PCR and subcloned into pEF5/FRT/V5-D-TOPO (Invitrogen) and stable SYF and SYF<sup>+/+</sup> cell lines generated using the Flp-In system (Invitrogen) according to the manufacturer's instructions.

### Immunoprecipitation

Cells were lysed in NP40 Buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 1 mM EDTA and 1% NP40). Anti-cortactin (4F11, 5 µg) was incubated with 0.5 mg clarified lysates for 2 hours at 4°C, then incubated with 40 µl Protein A/G Beads (Thermo Scientific) for 1 hour at 4°C. Immune complexes were collected by centrifugation, washed twice with NP40 Buffer, separated by SDS-PAGE and western blotted with antibodies as described.

### SH2 and PTB binding assay

SH2 and PTB domain binding assays were performed as described (Dierck et al., 2009; Machida et al., 2007). Briefly, SYF cell lysates were spotted in duplicate on a nitrocellulose membrane in register with the wells of a 96-well chamber plate. Each well was separately incubated with purified GST-SH2 or GST-PTB domains (~100 nM) for 2 hours. Probe binding was detected by enhanced chemiluminescence (ECL) (Perkin Elmer) and digitally captured (Kodak Image Station). Two independent experiments were performed in duplicate, providing four quantifiable data points for each probe. The array images were background-subtracted and the integrated density of each spot was measured using ImageJ (v1.40).

### Statistical analysis

Differences in mean values between groups were evaluated using a Student's *t*-test (two groups) or a one-way ANOVA (multiple groups) followed by Scheffé post-hoc testing.

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Supplementary material available online at

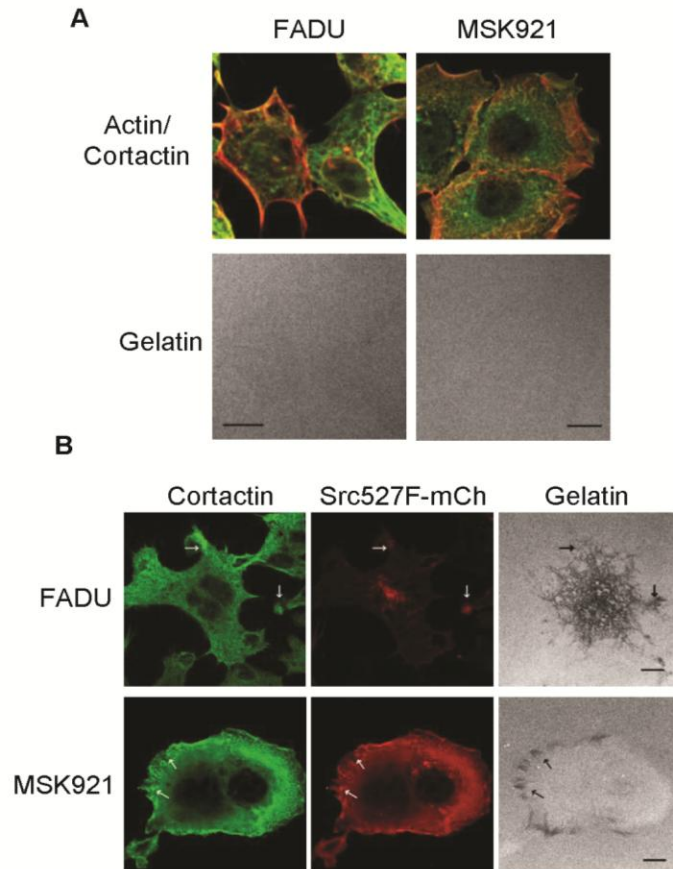
<http://jcs.biologists.org/cgi/content/full/123/22/3923/DC1>

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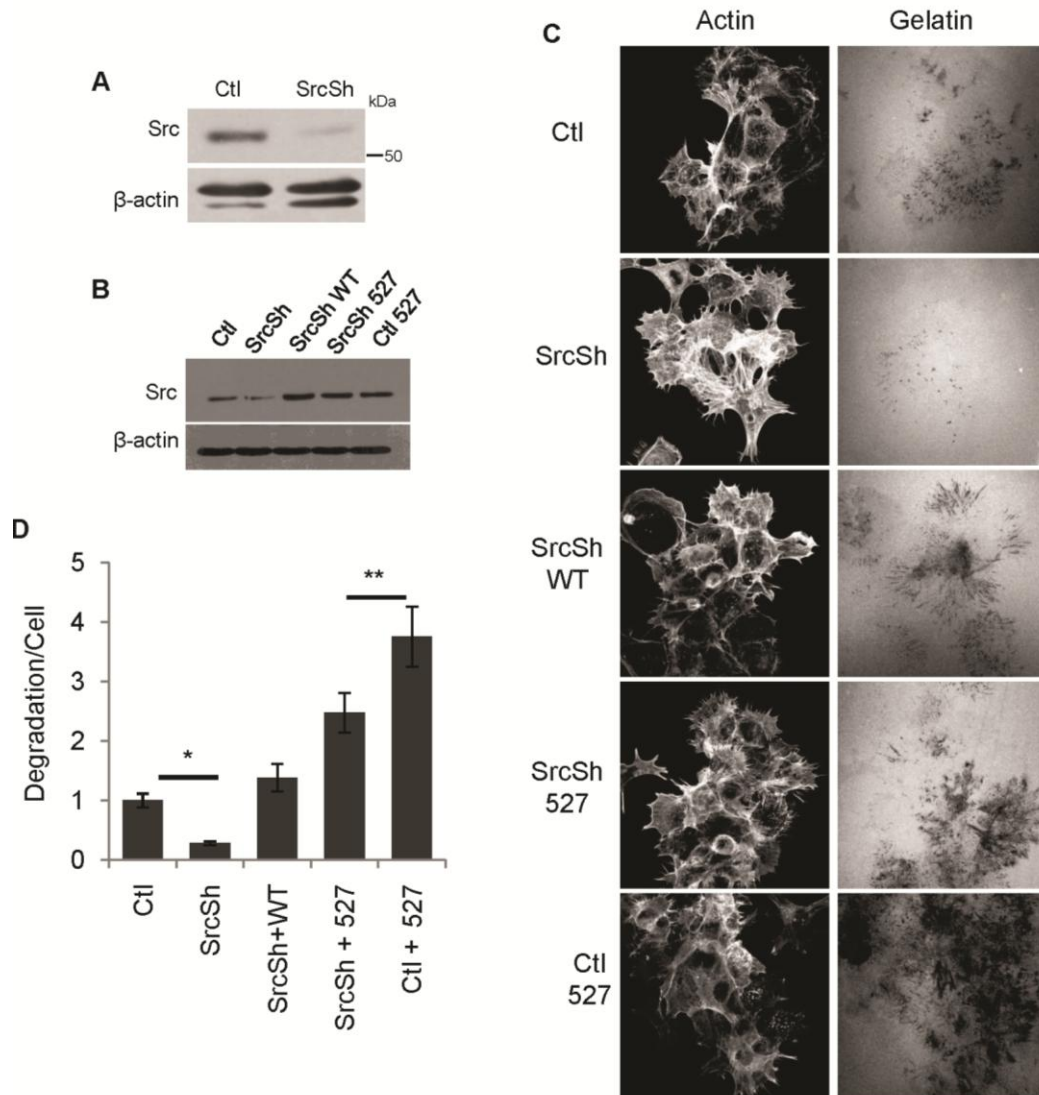
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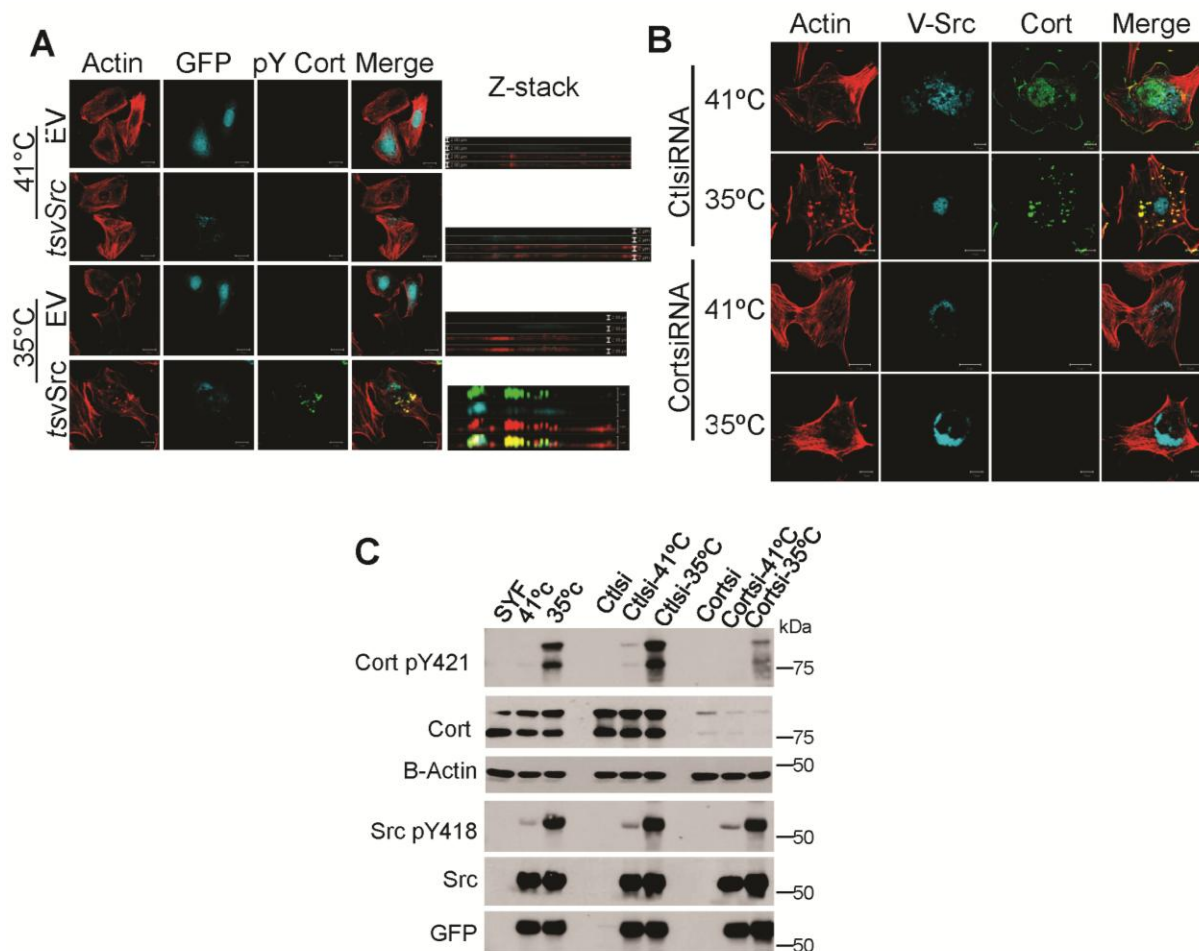
**Supplemental Figure 1** Confocal imaging of additional HNSCC cell lines assayed for Src activity and invadopodia-forming capability in Fig 1A. (A) The FADU and MSK921 HNSCC cell lines were incubated on FITC-coated gelatin (white) coverslips for 12 hours, then fixed and labeled with TRITC-phalloidin (red), and cortactin (green) (top panels). Both lines fail to make endogenous invadopodia or degrade matrix. (B) FADU and MSK921 cells transfected with Src527F-mCh (red) were incubated on FITC-coated gelatin (white) coverslips for 12 hours, then fixed and labeled with cortactin (green) (top panels). Src-induced invadopodia are present and identified by the cortactin aggregates (white arrows) that localize with Src527F and the dark holes in the FITC-gelatin (black arrows). Scale bars: 10  $\mu$ m.

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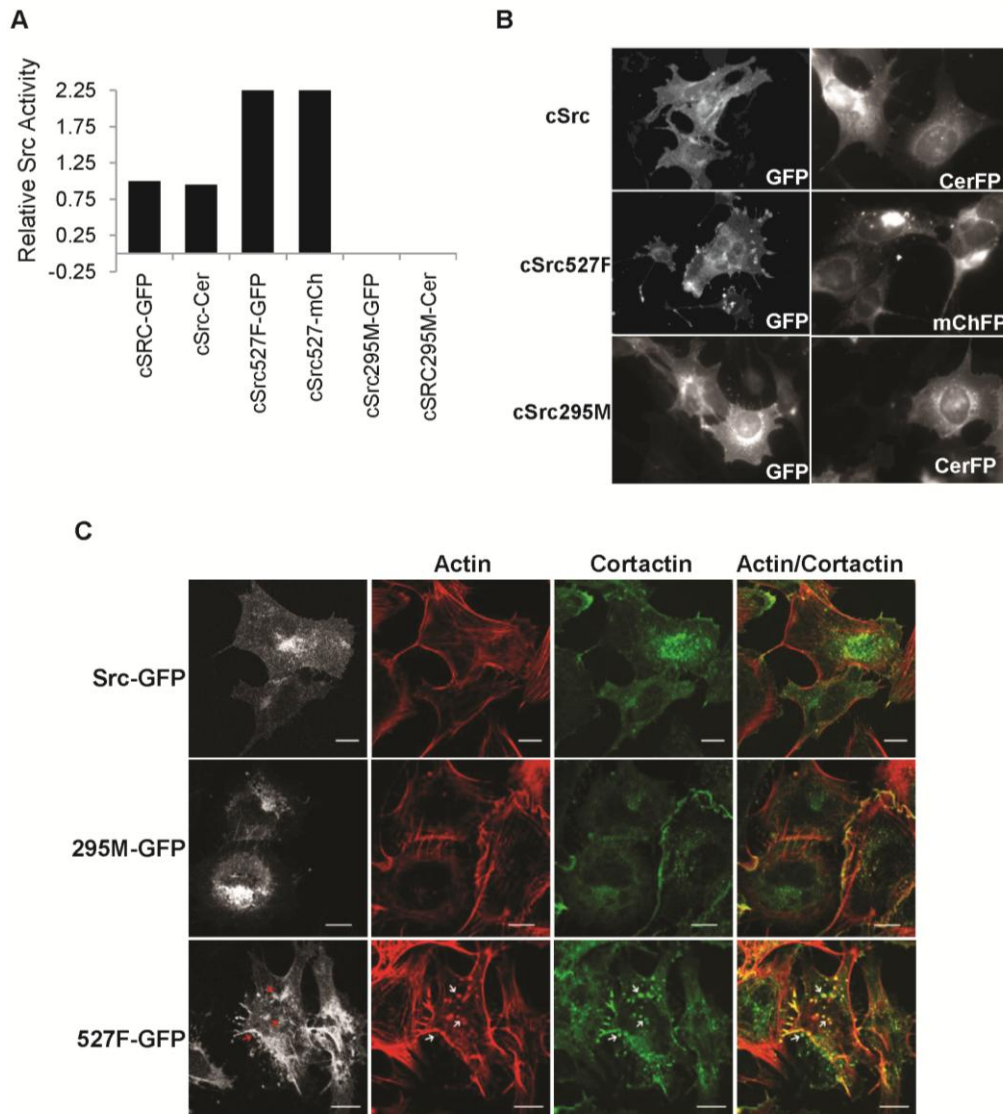
**Supplemental Figure 2** Src regulates invadopodia maturation in OSC19 cells. (A) Src protein levels in OSC19 cells treated with with Src shRNA (SrcSh) or a shRNA vector control (Ctl). Expression SrcWT (WT) or Src527F (527) in Ctl and SrcSh cells evaluated by immunoblotting (B) and confocal microscopy (C). Cells were plated on FITC-gelatin-coated (pseudocolored white) coverslips for 10 hours and immunolabeled with TRITC-phalloidin (red). Scale bars: 10  $\mu$ m. (D) The effect of Src expression on percentage of cells displaying invadopodia, the number of invadopodia per cell, and the amount of matrix degradation per cell were examined. Data are represented as mean  $\pm$  s.e.m., groups are statically different (\* $P$ <0.01, \*\* $P$ <0.05).

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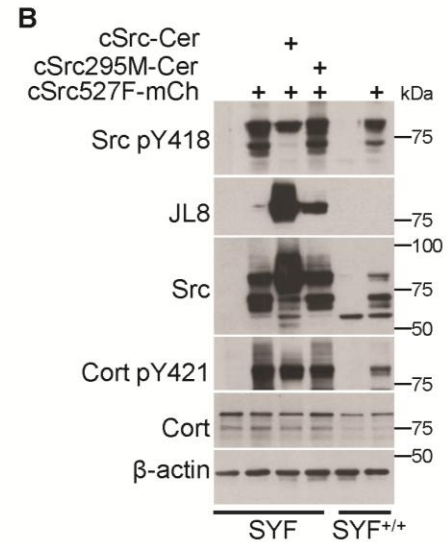
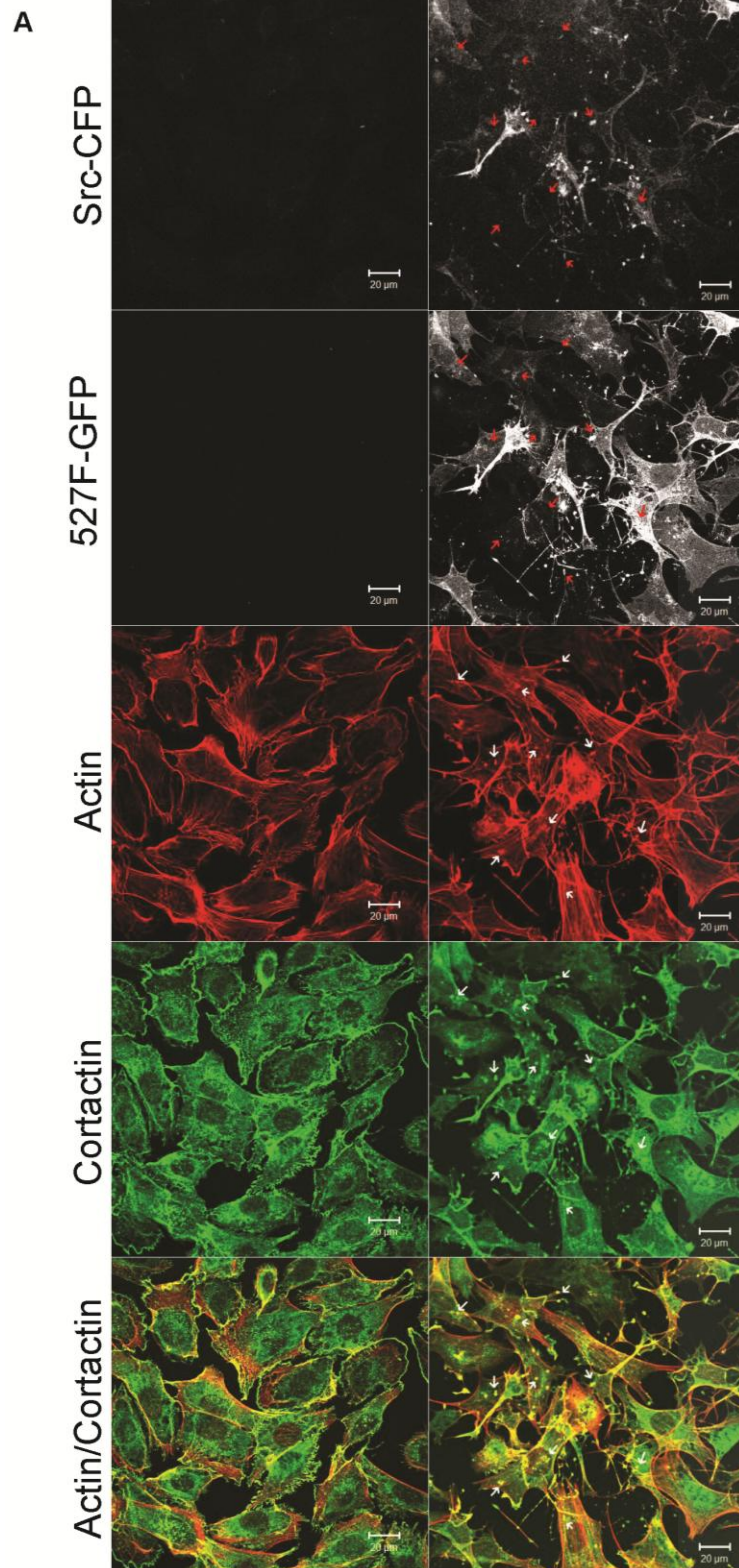
**Supplemental Figure 3** (A) Invadopodia are formed in cells expressing tsLa29-GFP at the permissive temperature. SYF cells transfected with empty GFP vector (EV) or with tsLa29 tagged with GFP on the C-terminus (tsLa29-GFP) were incubated at 41°C or 35°C. Cells were fixed, permeabilized, and dual-labeled with TRITC-phalloidin and a phospho-specific antibody for cortactin Tyr421. Cells were visualized by confocal microscopy through 2D and 3D (z-stack) sectioning. (B) Silencing of cortactin with siRNA prevents invadopodia formation. SYF cells were transfected with cortactin-targeted or control siRNA (Ctl) and incubated at 37°C for 2 days. Cells were then transfected with tsLa29-GFP and the experiment proceeded as described in A. Cells were fixed, permeabilized, and immunolabeled with TRITC-phalloidin and an anti-cortactin (4F11) antibody. Invadopodia fail to form in cells treated with cortactin siRNA. (C) Clarified cell lysates (30µg) from cells transfected with mock siRNA or siRNA to knock down cortactin, alone or in combination with tsLa29-GFP, were incubated at 41°C or 35°C, resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src (EC10), anti-cortactin (4F11), anti-cortactin-pY421, anti-GFP (JL8) and anti-β-actin antibodies. Scale bars: 10 µm.

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**Supplemental Figure 4** Characterization of fluorescent protein-tagged Src constructs. (A) Determination of relative Src kinase activity. SYF cells expressing Src-GFP, Src-cerulean, Src527F-GFP, Src527F-mCherry, Src295M-GFP, or Src295M-cerulean were lysed, resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src, and anti- $\beta$ -actin antibodies. Quantification of the relative Src phosphorylation in transfected SYF cells was conducted using densitometry. Src-pY18 protein expression was normalized to total Src protein levels. (B) Swept-field imaging of fluorescently labeled Src constructs. Fixed cells were imaged for expression and localization of GFP, mCherry, and cerulean-tagged Src fluorescent proteins by direct fluorescence. GFP, green fluorescent protein; CerFP, cerulean fluorescent protein; mChFP, mCherry fluorescent protein. (C) Confocal imaging of GFP-tagged SrcWT, Src295M, and Src527F. Transfected cells expressing the indicated Src constructs were fixed, permeabilized and immunolabeled with TRITC-phalloidin and the anti-cortactin (4F11) antibody. Arrows indicate invadopodia in cells expressing Src527F. Scale bars: 10  $\mu$ m. Kelley LC, et al. *J Cell Sci* 2010 Nov 15;123(Pt 22):3923-3932.





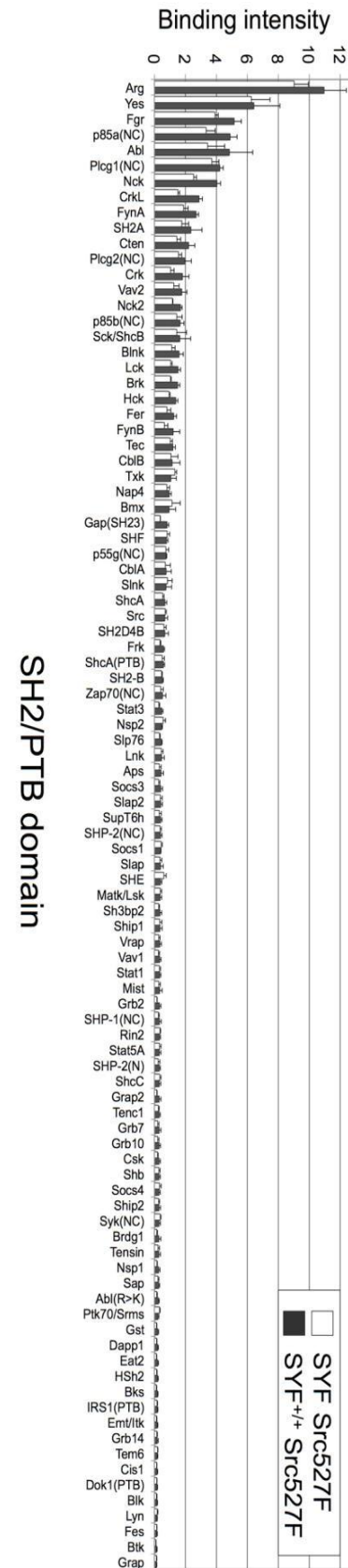
**Supplemental Figure 5** Co-transfection and localization of fluorescent protein tagged Src constructs. (A) Representative images of non-transfected SYF cells (left) and SYF cells co-expressing WT Src tagged with Cerulean fluorescent protein (Src-Cer) and GFP-tagged Src527F (527F-GFP) (right). Cells were fixed, permeabilized and immunolabeled with TRITC-phalloidin and anti-cortactin (4F11) antibody. Arrows denote invadopodia in the 527F-GFP-transfected cells. Scale bar: 20 $\mu$ m. (B) SYF and SYF<sup>+/+</sup> cells expressing cSrc527F-mCherry alone or in combination with Src-cerulean or Src295M-cerulean, were lysed and resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src, anti-GFP or anti-cerulean (JL8, does not recognize mCherry), anti-actin, anti-cortactin-pY421 and anti-cortactin (4F11) antibodies.

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## Supplemental Figure 6

Comprehensive SH2 and PTB binding assay. An in vitro binding assay was performed using 91 GST SH2 domains and three GST PTB domains, representing nearly the full complement of human phosphotyrosine-binding domains. GST and mutated Abl SH2 domain (Abl R>K) were used as negative controls. SH2 binding to SYF cell lysate was determined by densitometric quantification of digitally captured chemiluminescence images. The mean raw binding intensities with s.e.m. from two independent experiments are shown. Domains are ordered from left to right by their binding intensity to SYF<sup>+/+</sup> cells expressing Src527F to reflect rank order relevance for invadopodia maturation.

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## Karen E. Hayes

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| EDUCATION                | West Virginia University School of Medicine<br>Morgantown, WV<br>PhD candidate, Cancer Cell Biology  | 2004-present |
|                          | Edinboro University of Pennsylvania<br>Edinboro, PA<br>B. S., Biology, Cum Laude, Department of Biology  | 1997         |
| RELATED<br>EMPLOYMENT    | Free-Col Laboratories, A Division of Modern<br>Industries, Meadville, PA<br>Laboratory Analyst   | 1999-2004    |
|                          | Areas of responsibilities include maintaining bioassay cultures, reference toxicant analysis, and WET test analysis, wet chemistry analysis utilizing spectrophotometer, ion selective electrodes, titrations and distillation units. Other responsibilities included assisting in the microbiology department and reviewing other chemist's analytical reports.   |              |
| DOCTORAL<br>DISSERTATION | Abelson Kinase Based Regulation of Tumor Cell Invasion in<br>HNSCC   |              |
|                          | <i>Dissertation Defense Date: December 11, 2012</i>  |              |
|                          | Laboratory techniques learned and utilized included biochemical protein analysis and purification (Western blotting, immunoprecipitation, affinity precipitation, recombinant protein production, gelatin zymography, ELISA); cellular migration, adhesion, and invasion assays (ECIS, wound healing assays, gelatin degradation assay, Boyden chamber based assays, 3D-Collagen I invasion assay); kinase assays; DNA cloning/mutagenesis; fluorescent/confocal microscopy; flow cytometry; tissue/cell culture techniques (maintaining cell lines, cellular transfection, lentivirus preparation and subsequent cellular |              |

infection); and orthotopic xenografts of oral squamous cell carcinoma examining perineural invasion and lymph node metastasis.

## PUBLICATIONS

Ableson Kinases Negatively Regulate Invadopodia Function and Invasion in Head and Neck Squamous Cell Carcinoma by Inhibiting an HB-EGF Autocrine Loop, **Karen E. Hayes\***, Elyse L. Walk, Amanda Gatesman Ammer, Laura C. Kelley, Karen H. Martin, and Scott A. Weed *Oncogene* 2012 Nov; 1(2) online pub doi.1038/onc.2012.513.

Quantitative Measurement of Invadopodia-mediated Extracellular Matrix Proteolysis in Single and Multicellular Contexts, Karen H. Martin\*, **Karen E. Hayes**, Elyse L. Walk, Amanda Gatesman Ammer, Steven M. Markwell and Scott A. Weed *J. Vis Exp. (JOVE)* 2012 Aug 27;66.

Multi-photon Imaging of Tumor Cell Invasion in an Orthotopic Mouse Model of Oral Squamous Cell Carcinoma, Gatesman Ammer A\*, **Hayes KE**, Martin KH, Zhang L, Spirou GA, and Weed SA. *J. Vis Exp. (JOVE)* 2011 Jul; 53:2941.

Revisiting the ERK/Src Cortactin Switch, **Hayes KE\***, Kelley LC\*, Ammer AG\*, Martin KH, and Weed SA *Commun. Integr. Biol.* 2011 Mar;4(2):205-207.

Further Insights into Cortactin Conformational Regulation, Jason V. Evans\*, Laura C. Kelley, **Karen E. Hayes**, Amanda Gatesman Ammer, Karen H. Martin and Scott A. Weed *Bioarchitecture* 2011 Jan;1(1):21-23.

Oncogenic Src Requires a Wild-type Counterpart to Regulated Invadopodia Maturation, Kelley LC\*, Ammer AG, **Hayes KE**, Martin KH, Machida K, Jia L, Mayer BJ, and Weed SA *J. Cell Sci.* 2010 Nov 15;123:3923-3932.

Cortactin Phosphorylated by ERK1/2 Localizes to Sites of Dynamic Actin Regulation and Is Required for Carcinoma Lamellipodia Persistence, **Karen E. Hayes\***, Laura C. Kelley\*, Amanda Gatesman Ammer\*, Karen H. Martin, and Scott A. Weed *PLoS One* 2010 Nov;5(11):e13847.

Sarcatinib Impairs Head and Neck Squamous Cell Carcinoma Invasion by Disrupting Invadopodia Function, **Hayes KE\***, Ammer AG\*, Kelley LC\*, Evans JV, Lopez-Skinner LA, Martin KH,



Frederick B, Rothschild BL, Raben D, Elvin P, Green TP, Weed SA  
*J Cancer Sci Ther.* 2009 Nov 30;1(2):52-61.

\* Denotes First Authors

## ABSTRACTS

The 4<sup>th</sup> Biennial National IDeA Symposium, Washington, DC, June 2012, *Ableson Kinases Negatively Regulate Invadopodia and Invasion in HNSCC*, **Karen Hayes** and Scott Weed.

The American Society for Cell Biology 50th Annual Meeting, Philadelphia, PA, December 2010, *c-Abl is a Novel Negative Regulator of Invadopodia Activity*, **Karen Hayes**, Karen H. Martin, Laura Kelley, Mark Culp and Scott Weed.

EJ Van Liere Convocation and Research Day, Morgantown, WV, April 2009, *A c-Abl -Cortactin Complex Regulates Invadopodia Activity*, **Karen E. Hayes** and Scott A. Weed.

4th Annual West Virginia COBRE/INBRE Conference, Morgantown, WV, October 2008, *A c-Abl-Cortactin Complex Regulates Invadopodia Activity*, **Karen E. Hayes**, Karen H. Martin and Scott A. Weed.

The 3rd Annual West Virginia COBRE/INBRE Conference, Charleston, WV, November 2007, *Role of c-Abl in Cortactin Phosphorylation*, **Karen E. Hayes**, Laura F. Gibson, Mark Auble, Ann Marie Pendergast, Scott A. Weed.

EJ Van Liere Convocation and Research Day, Morgantown, WV May 2007, *Role of c-Abl in Cortactin Phosphorylation*, **Karen E. Hayes**, Laura F. Gibson, Mark Auble, Ann Marie Pendergast, Scott A. Weed.

The American Association for Cancer Research 98th Annual Meeting, Los Angeles, CA, April 2007, *Role of c-Abl in Cortactin Phosphorylation*, **Karen E. Hayes**, Laura F. Gibson, Mark Auble, Ann Marie Pendergast, Scott A. Weed.

## PROFESSIONAL DEVELOPMENT

Organized and hosted guest speaker, Dr Donna Webb, for West Virginia University Cell Biology Training Seminar, May 2010.

Imaging Ethics, Acquisition, Post-Processing and Quantification Course, taught by Jerry Sedgewick, June 2012.

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|-------------|---|-----------|
| MEMBERSHIPS | AACR (American Association for Cancer Research)           | 2010-2012 |
|             | ASCB (American Society for Cell Biology)                  | 2006-2012 |
|             | West Virginia University Cytoskeletal Signaling Group     | 2008-2010 |
|             | West Virginia University Cell Biology Training Consortium |           |
|             |   | 2009-2012 |